UNIVERSIDAD COMPLUTENSE DE MADRID FACULTAD DE CIENICAS QUÍMICAS
Departamento de Bioquímica y Biología Molecular

TESIS DOCTORAL

Mecanismos de neurotoxicidad en el circuito corticoestrial: papel protector del receptor CB1 cannabinoide

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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RESUMEN/SUMMARY

Resumen

Los ganglios basales son una serie de núcleos subcorticales interconectados que incluyen el estriado (caudado y putamen en primates), el globo pálido (segmentos internos y externos), el núcleo subtalámico y la sustancia nigra (*pars reticulata* y *pars compacta*). Se trata de un nodo fundamental para muchos procesos conductuales y neurobiológicos como el control afectivo, la actividad motora y las funciones cognitivas. La mayoría (~ 95%) de las neuronas del estriado son neuronas GABAérgicas espinosas medianas (del inglés *medium spiny neurons*, MSNs), que reciben aferencias glutamatérgicas principalmente de la corteza y el tálamo. Está bien establecido que existen dos poblaciones principales de MSNs: las MSNs de la vía directa, que proyectan principalmente a la sustancia nigra *pars reticulata* y el globo pálido interno, y expresan receptores de dopamina de tipo 1 (D1R-MSNs), y las MSNs de la vía indirecta, que proyectan principalmente al globo pálido externo y expresan receptores de dopamina de tipo 2 (D2R-MSNs). Dada su heterogeneidad neuroquímica y funcional, no es de extrañar que el desequilibrio entre la MSNs de la vía directa e indirecta subyazca a la patogenia de diversos trastornos de los ganglios basales como la enfermedad de Huntington (EH) y la enfermedad de Parkinson (EP). Se han descrito cambios notables en la actividad electrofisiológica de las D1R-MSNs, más que de las D2R-MSNs, en modelos de EH durante diferentes etapas de la enfermedad, lo que sugiere un papel importante de esta población neuronal en el desarrollo y progresión de la patología. Por otra parte, en pacientes y modelos animales de EH se ha observado una pérdida selectiva de D2R-MSNs.

Muchos modelos conceptuales apoyan la hipótesis de que estas dos poblaciones de MSNs son mecánica y funcionalmente opuestas. Sin embargo, la obtención de evidencias empíricas que demuestren sus funciones ha sido difícil, debido a que ambas poblaciones celulares se encuentran físicamente entremezcladas y son morfológicamente indistinguibles. La aplicación de la optogenética, para controlar la actividad neuronal con exquisita resolución temporal a través de la ingeniería de opsinas, ha proporcionado una plataforma de expansión para descifrar las funciones de estriado. Más recientemente, los receptores de diseño activados exclusivamente por drogas de diseño (DREADDs) han sido desarrollados para controlar remotamente la actividad neuronal. Esta poderosa herramienta se basa en la modificación molecular de los receptores metabotrópicos humanos, principalmente muscarínicos, lo que conduce a una ínfima potencia y eficacia en su interacción con el ligando natural (acetilcolina) y a una elevadísima potencia y eficacia de moléculas como la clozapina-*N*-óxido (CNO), de alta biodisponibilidad por vía oral y farmacológicamente inertes. Es asimismo destacable que los DREADDs carecen de actividad constitutiva y de procesos de desensibilización detectables *in vitro* e *in vivo*, ofreciendo por tanto un complejo receptor-efector atractivo para modificar la actividad celular remotamente mediante la manipulación de las vía de señalización dependientes de proteínas Gi, Gq y Gs. El uso de animales transgénicos, o su expresión mediante virus recombinantes adeno-asociados (rAAV), permite expresar un DREADD en una población celular particular de una región determinada del cerebro. La administración de CNO a estos animales mutantes ha demostrado la capacidad de cambiar patrones conductuales y electrofisiológicos de forma dependiente de la población celular y la vía molecular involucrada. Curiosamente, al expresar DREADD-Gq en neuronas principales, altas dosis de CNO son incluso capaces de inducir eventos excitotóxicos. Así, este sistema permite un control remoto, específico y reversible de la actividad neuronal en poblaciones celulares específicas, siendo adecuado para estudios tanto a corto como a largo plazo.

La señalización dependiente de receptores metabotrópicos es absolutamente necesaria para el correcto funcionamiento del estriado. Así, numerosos neurotransmisores/neuromoduladores como dopamina, glutamato, adenosina, acetilcolina y endocannabinoides controlan la actividad y la plasticidad de las MSNs a través de diversas familias de receptores acoplados a proteínas G (GPCRs). En concreto, los principales receptores de dopamina presentes en las MSNs, D1R y D2R, están acoplados a proteínas Gs/olf y Gi, respectivamente, y se han descrito detalladamente sus mecanismos de señalización en el estriado. Los receptores acoplados a Gq, como los receptores metabotrópicos de glutamato mGlu1/5 y los receptores muscarínicos de acetilcolina M1/3/5, son también muy importantes en el control de la excitabilidad de las MSNs, habiéndose descrito una sobre-activación de la señalización dependiente de ellos en algunos modelos de enfermedades relacionadas con los ganglios basales, como la EH y la adicción a drogas. Sin embargo, hasta ahora no se ha dilucidado el impacto preciso y el modo de acción de esta señalización a través de proteínas Gq en las MSNs.

Una pregunta sin respuesta en la mayoría de las enfermedades neurodegenerativas es qué factores precisos dictan el daño selectivo de una población neuronal determinada. Específicamente en la EH, una enfermedad neurodegenerativa devastadora donde las estructuras principalmente afectadas son el estriado y la corteza, no se conocen enteramente los mecanismos que hacen que las neuronas más abundantes en el estriado (las MSNs) sean tan vulnerables. Durante muchos años, se han acumulado evidencias que apoyan la implicación en la progresión de la EH de una desregulación progresiva de las redes neuronales que enlazan la corteza y el estriado. En particular, la hipótesis de la excitotoxicidad de la EH sostiene que la neurodegeneración estriatal está mayoritariamente causada por un exceso de impulsos excitadores corticales deletéreos, debido a factores como la liberación masiva de glutamato en los terminales corticoestriatales y al consiguiente aumento en la entrada de Ca^{2+} a través de los receptores ionotrópicos de glutamato en las MSNs. En la actualidad, no existe ningún tratamiento neuroprotector o curativo para la EH, y ésta representa además el modelo más estudiado y representativo de las enfermedades neurodegenerativas producidas por expansiones de un triplete, como es el caso, por ejemplo, de algunas ataxias. Por lo tanto, descubrir el mecanismo patogénico subyacente a la EH y encontrar un tratamiento eficaz desde el punto de vista de la neuroprotección o erradicación podría tener implicaciones inmediatas para cualquier otra enfermedad neurodegenerativa causada por expansiones de un triplete. Un número importante de estudios ha analizado la expresión y el papel del sistema endocannabinoide en la EH. De hecho, como se expone más adelante, la EH constituye uno de los mejores modelos de enfermedad actualmente disponibles para evaluar la relevancia fisiopatológica y terapéutica del sistema endocannabinoide en las enfermedades neurodegenerativas.

La planta del cáñamo (*Cannabis sativa* L.) y sus derivados se han utilizado en medicina desde hace al menos cincuenta siglos. Sin embargo, la estructura química de sus componentes activos (los cannabinoides) no fue aclarada hasta la década de 1960. Tres décadas más fueron necesarias para la identificación en el organismo de receptores específicos de cannabinoides. Hasta ahora, dos receptores de cannabinoides han sido bien caracterizados: el de tipo 1 (CB1R), que es especialmente abundante en las áreas del sistema nervioso central implicadas en el control de la actividad motora (ganglios basales, cerebelo), aprendizaje y memoria (corteza, hipocampo), emociones (amigdala), percepción sensorial (tálamo) y varias funciones autónomas y endocrinas (hipotálamo, médula), y el de tipo 2 (CB2R), que se expresa preferentemente en las células (linfocitos B y T macrófagos) y tejidos (bazo, ganglios linfáticos) del sistema inmune, aunque también está presente en algunas células del sistema nervioso, mayoritariamente en microglía. Las moléculas endógenas que interaccionan con los receptores cannabinoides, los endocannabinoides (anandamida y 2-araquidonilglicerol), también han sido aislados. La generación de endocannabinoides se produce "bajo demanda" a partir de precursores de lípidos de la membrana plasmática y está estrechamente controlada por la actividad neuronal. La señalización endocannabinoide proporciona un importante mecanismo de retroalimentación para atenuar la excesiva actividad presináptica y, así, sintoniza la funcionalidad y la plasticidad de muchas sinapsis, especialmente glutamatérgicas y GABAérgicas. Junto con esta función neuromoduladora, estudios en diversos modelos animales apoyan que CB1R desempeña un papel importante en la supervivencia neuronal en entornos fisiopatológicas como la lesión cerebral aguda y situaciones de neuroinflamación. Sin embargo, la evaluación de la relevancia fisiológica y el potencial terapéutico del CB1R en las enfermedades neurológicas se ve impedida, al menos en parte, por la falta de conocimiento de la especificidad celular de acción de CB1R. Entre todas las enfermedades neurodegenerativas, la EH constituye uno de los mejores modelos disponibles actualmente para el estudio del potencial neuroprotector de CB1R debido a varias razones:

(I) CB1R es uno de los GPCRs más abundantes en el cerebro; en concreto, se expresa en gran cantidad en los ganglios basales, en las sinapsis establecidas por neuronas que contienen GABA (especialmente MSNs, las células que degeneran principalmente en la EH) o glutamato (especialmente neuronas de proyección corticoestriatales, que controlan críticamente la función de las MSNs) como transmisores, y desempeña un papel clave en el control del comportamiento motor (uno de los procesos más característicamente alterados en la EH).

(II) En pacientes con EH y en modelos animales de la enfermedad existe una disminución selectiva, notable y con un gradiente dorsolateral de CB1R en los ganglios basales. Además, esta pérdida de CB1R parece reflejar, al menos en parte, el patrón de daño de neuronas GABAérgicas característico de la enfermedad.

(III) La antedicha pérdida de CB1R ocurre en etapas tempranas de HD y antes de la aparición de síntomas clínicos manifiestos, neurodegeneración y cambios bruscos en otros parámetros neuroquímicos.

(IV) En el contexto del bien establecido papel neuroprotector de los cannabinoides, nuestro grupo y otros han demostrado previamente un papel neuroprotector de CB1R en modelos transgénicos de ratón de la EH.

En este contexto, el **OBJETIVO GLOBAL** de esta Tesis Doctoral comprende el estudio de los mecanismos de la vía directa e indirecta implicados en la disfunción corticoestriatal y la neurodegeneración, así como el emplazamiento neurobiológico selectivo de la acción neuroprotectora del CB1R en dicho contexto.

Este objetivo global se puede dividir en 2 **OBJETIVOS ESPECÍFICOS**:

Objetivo 1. Examinar los mecanismos moleculares y la relevancia fisiopatológica de la señalización dependiente de proteínas Gq en las MSNs de la vía directa e indirecta del estriado dorsal.

Objetivo 2. Estudiar el papel de las diferentes poblaciones de CB1R, es decir, los situados en las neuronas GABAérgicas (MSNs), glutamatérgicas (neuronas de proyección corticoestriatal) o astrocitos, en la vulnerabilidad diferencial de las MSNs de la vía directa e indirecta del estriado dorsal.

Los **RESULTADOS** obtenidos se han dividido en dos bloques:

En el **primer bloque de Resultados**, utilizando diferentes aproximaciones experimentales, especialmente la tecnología DREADD, demostramos que la activación sostenida de la señalización a través de proteínas Gq deteriora la funcionalidad de las MSNs y desvelamos el mecanismo molecular exacto que subyace a este proceso, que consiste en el eje de señalización PLC/Ca^{2+}/PYK2/JNK. Además, esta ruta de señalización intracelular se encuentra implicada en el control del funcionamiento del estriado dorsal de ratón *in vivo*, como se demuestra a través de la alteración de la integridad neuronal y el comportamiento. Así, la activación aguda de la señalización por Gq en las MSNs de la vía directa o vía indirecta produce un aumento o una disminución, respectivamente, de parámetros dependiente de actividad. Por el contrario, la activación continua de la señalización por Gq deteriora la funcionalidad de las MSNs de la vía directa y vía indirecta y altera el comportamiento, y el patrón electroencefalográfico relacionado con la actividad, controlados por ambos circuitos neuroanatómicos.

En el **segundo bloque de Resultados**, mediante el empleo de un conjunto de estrategias genéticas, quimiogenéticas y farmacológicas destinadas a manipular la función del CB1R de forma espaciotemporalmente restringida *in vivo*, mostramos que la población de CB1R situada en las proyecciones corticoestriatales, mediante la disminución de la liberación de glutamato, protege selectivamente las MSNs de la vía directa del estriado dorsal del ratón frente al daño inducido por la expresión de huntingtina mutante (mtHtt) en la corteza. En concreto, la expresión de mtHtt en la corteza motora daña las D1R-MSNs pero no las D2R-MSNs (i) cuando CB1R se bloquea farmacológicamente con rimonabant o (ii) cuando CB1R se inactiva genéticamente de forma condicional en neuronas corticales principales. Este proceso neurotóxico se rescata mediante la administración de MK-801, un antagonista del receptor NMDA. Asimismo, se observa una mayor vulnerabilidad selectiva de las D1R-MSNs frente a las D2R-MSNs cuando se sobreactivan las proyecciones glutamatérgicas corticoestriatales mediante el uso de DREADD y CB1R se encuentra farmacológicamente bloqueado. El *pool* de CB1R situado en las proyecciones corticoestriatales, mediante la inhibición de la transmisión glutamatérgica, también es capaz de proteger las MSNs del daño derivado de la expresión de mtHtt en astrocitos estriatales. Por último, la MGL (enzima responsable de la degradación del 2-araquidonilglicerol) controla desde los astrocitos la disponibilidad del 2-araquidonilglicerol involucrado en la protección de las MSNs.

Los resultados obtenidos en esta tesis nos permiten obtener las siguientes **CONCLUSIONES**:

I. Los circuitos del estriado dorsal pueden ser "activados" por la señalización aguda de proteínas Gq o "apagados" por la señalización sostenida de proteínas Gq. Específicamente, la activación sostenida de la señalización por Gq inactiva las MSNs del estriado dorsal a través de una vía intracelular que converge en JNK.

II. La población de CB1R situada en las proyecciones corticoestriatales, al menos en gran parte mediante la inhibición de la liberación de glutamato, protege selectivamente las D1R-MSNs del estriado dorsal frente al daño cortical producido por excitotoxicidad o mtHtt.

Colectivamente, estos resultados definen el mecanismo molecular y señalan la relevancia funcional de la señalización dependiente de proteínas Gq en los circuitos estriatales, en estados normales y de sobreactivación, y definen al *pool* de CB1R cortical como uno de los elementos neuroquímicos implicados en la diferente vulnerabilidad que presentan las D1R-MSNs frente a las D2R-MSNs en la EH. Todo ello puede contribuir a entender el papel de una señalización cannabinérgica-glutamatérgica coordinada en el control de las vías directa e indirecta corticoestriatales y su desregulación en enfermedades que afectan a los ganglios basales.

SUMMARY

The basal ganglia are a series of interconnected subcortical nuclei including the striatum (caudate and putamen in primates), the globus pallidus (internal and external segments), the subthalamic nucleus, and the substantia nigra (pars reticulata and pars compacta). They are a key node for many behavioural and neurobiological processes such as motor activity, cognitive functions, and affective control. The vast majority (~95%) of neurons within the striatum are GABAergic medium spiny neurons (MSNs), which receive glutamatergic inputs primarily from the cortex and from specific thalamic nuclei. It is well established that there are two major populations of MSNs: direct pathway MSNs, that project mainly to the substantia nigra pars reticulata and express dopamine type 1 receptors (D1R-MSNs), and indirect pathway MSNs, that project mainly to the external globus pallidus and express dopamine type 2 receptors (D2R-MSNs). Given their neurochemical and functional heterogeneity, it is not surprising that the imbalance between direct and indirect pathway's MSNs may underlie the pathogenesis of several basal ganglia disorders as Huntington's disease (HD) and Parkinson´s disease (PD). Remarkable changes in the electrophysiological activity of D1R-MSNs, rather than of D2R-MSNs, have been shown in mouse models of HD at different disease stages, pointing out to an important role of the former neuronal population. On the other hand, a somewhat selective loss of indirect pathway's MSNs has been observed in HD patients and animal models.

Many conceptual models hypothesize that those two MSN populations oppose one another both mechanistically and functionally. However, obtaining empirical evidence to support their roles has proven difficult because these cell populations are physically intermingled and morphologically indistinguishable. The implementation of optogenetics to control neuronal activity with exquisite temporal resolution using engineered opsins has provided an expanding platform for decoding striatal functions. More recently, the designer receptor exclusively activated by designer drug (DREADD) technology has been developed for controlling neuronal activity remotely. This powerful tool is very frequently based on the molecular evolution of human muscarinic receptors, leading to reduced potency and efficacy of the native ligand (acetylcholine) and high potency and efficacy of the orally bioavailable, pharmacologically inert ligand clozapine-*N*-oxide (CNO). Importantly, DREADDs lack detectable constitutive activity and desensitization processes *in vitro* and *in vivo*, thus providing an attractive receptor-effector complex to modify cellular activity remotely by manipulating Gi-, Gq- and Gs-coupled pathways. The use of transgenic animals and/or recombinant adeno-associated virus (rAAV)-mediated gene delivery allows to express the DREADD in a particular cell population of a given brain region. Administration of CNO to these mutant animals has been shown to strongly change behavioural and electrophysiological patterns according to the cell population and molecular pathway involved. Interestingly, by engaging DREADD-Gq expressed in principal neurons, high doses of CNO are even able to induce excitotoxic seizures. Thus, this system allows a specific and reversible remote control of neuronal activity in specific cell populations and is suitable for long-term studies.

Metabotropic signalling is absolutely necessary for the proper functioning of the striatum. Neurotransmitters/neuromodulators such as dopamine, glutamate, adenosine, acetylcholine, and endocannabinoids control the activity and plasticity of MSNs by engaging various G protein-coupled receptor (GPCR) families. Specifically, the main dopamine receptors present in MSNs, namely D1R and D2R, are coupled to Golf and Gi proteins, respectively, and the detailed roles of these signalling axes on striatal functions have been reported. Gq-coupled receptors such as metabotropic glutamate mGlu1/5 receptors and muscarinic acetylcholine M1/3/5 receptors are also very important in the control of MSN excitability, and an overactive Gq-protein-driven signalling has been shown to occur in various models of basal ganglia-related diseases such as HD and drug addiction. However, the precise impact and mode of action of Gq-protein signalling on MSNs have not been clarified so far.

A key unanswered question in most neurodegenerative diseases is what precise factors dictate the selective damage of a particular neuronal population. Specifically in HD, a devastating neurodegenerative disease where the primarily affected structures are the cortex and striatum, the mechanisms by which striatal principal neurons (MSNs) are so highly vulnerable are incompletely understood. For many years, evidence has accumulated supporting that a progressive dysregulation of the neuronal networks linking deep-cortical and striatal pathways is involved in HD pathology. Particularly, the excitotoxicity hypothesis of HD supports that striatal neurodegeneration is majorly caused by an excess of deleterious cortical excitatory inputs due to factors such as massive glutamate release from corticostriatal terminals and overactivation of ionotropic glutamate receptors ad Ca^{2+} influx in MSNs. At present, no neuroprotective or curative treatment exists for HD, and it represents the most studied model and the most prevalent

of the neurodegenerative diseases produced by triplet expansions, to which some ataxias also belong. Therefore, discovering the pathogenic mechanism underlying HD, and finding an effective treatment from the point of view of neuroprotection or eradication, could thus have immediate implications for any other neurodegenerative disease caused by triplet expansions. A significant number of studies have dealt with the expression and role of the endocannabinoid system in HD. As a matter of fact, as discussed below, HD constitutes one of the best currently available model disease to assess the pathophysiological relevance and therapeutic potential of the endocannabinoid system in neurodegenerative diseases.

The hemp plant (*Cannabis sativa* L.) and its derivatives have been used in medicine for at least fifty centuries. However, the chemical structure of their active components (the cannabinoids) was not elucidated until the early 1960s. Three decades more were necessary for the identification in our body of specific cannabinoid receptors to be achieved. So far, two cannabinoid receptor types have been well characterized: CB1R, which is especially abundant in areas of the central nervous system involved in the control of motor activity (basal ganglia, cerebellum), learning and memory (cortex, hippocampus), emotions (amygdala), sensory perception (thalamus) and various autonomic and endocrine functions (hypothalamus, medulla), and CB2R, which is preferentially expressed in cells (B and T lymphocytes, macrophages) and tissues (spleen, lymph nodes) of the immune system, although it is also present in the nervous system, mainly in microglia. Endogenous molecules that engage cannabinoid receptors, the so-called endocannabinoids (anandamide and 2-arachidonoylglycerol), have also been isolated. Endocannabinoid generation occurs by "on-demand" synthesis and cleavage of plasma membrane lipid precursors, and is tightly controlled by neuronal activity. Endocannabinoid signalling serves as a major feedback mechanism to prevent excessive presynaptic activity and thus tunes the functionality and plasticity of many synapses, especially glutamatergic and GABAergic. In concert with this neuromodulatory function, studies in various animal models support that the CB1R plays an important role in the promotion of neuron survival in pathophysiological settings such as acute brain injury and neuroinflammatory conditions. However, the assessment of the physiological relevance and therapeutic potential of CB1R in neurological diseases is hampered, at least in part, by the lack of knowledge of the cell-population specificity of CB1R action. Among all the neurodegenerative diseases, HD constitutes one of the best currently available models to study the neuroprotective potential of CB1R due to several reasons:

(I) CB1R is one of the most abundant GPCRs in the brain; specifically, it is highly expressed in the basal ganglia at synapses established by neurons containing GABA [especially MSNs, the cells that primarily degenerate in HD] or glutamate (especially corticostriatal projecting neurons, which critically control MSNs function) as transmitters, and play a key role in the control of motor behaviour (one of the processes that is most characteristically affected in HD).

(II) A remarkable and dorsolaterally-selective down-regulation of CB1R has been documented in the basal ganglia of HD patients and animal models, and, of interest, this loss of CB1R seems to reflect, at least in part, the GABAergic-neuron damage pattern characteristic of the disease.

(III) The aforementioned loss of CB1R occurs at early stages of HD and prior to the appearance of overt clinical symptoms, neurodegeneration and bulk changes in other neurochemical parameters.

(IV) In the context of the well-established neuroprotective role of cannabinoids, our group and others have demonstrated a neuroprotective role of CB1R in transgenic mouse models of HD.

In this context, the **GLOBAL AIM** of this Doctoral Thesis embraces the study of direct-pathway versus indirect-pathway mechanisms involved in corticostriatal dysfunction and neurodegeneration, as well as the selective neurobiological site(s) of CB1R neuroprotective action within the corticostriatal circuitry.

This main objective can be divided into 2 **SPECIFIC AIMS**:

Objective 1. To examine the molecular mechanisms and physiopathological relevance of Gq-protein signalling in D1R-MSNs and D2R-MSNs within the dorsal striatum.

Objective 2. Study the role of different CB_1R pools, namely those situated on GABAergic neurons (MSNs), glutamatergic neurons (corticostriatal projection neurons) or astrocytes, in the differential vulnerability of D1R-MSNs and D2R-MSNs within the dorsal striatum.

The **RESULTS** obtained have been divided into two blocks:

In the **first block of Results**, by using different experimental approaches, especially the DREADD technology, we show that sustained activation of Gq-protein signaling impairs the functionality of striatal neurons and unveil the precise molecular mechanism underlying this process, which involves a PLC/Ca^{2+}/PYK2/JNK pathway. Moreover, engagement of this intracellular signaling route is functionally active in the mouse dorsal striatum *in vivo*, as proven by the disruption of neuronal integrity and behavioral tasks. Acute Gq-protein activation in direct-pathway or indirect-pathway neurons produces an enhancement or a decrease, respectively, of activity-dependent parameters. In contrast, sustained Gq-protein activation impairs the functionality of direct-pathway and indirect-pathway neurons and disrupts the behavioral performance and electroencephalography-related activity tasks controlled by either anatomical framework.

In the **second block of Results**, by using an array of genetic, chemogenetic and pharmacological strategies to manipulate cannabinoid CB1R function in a spatiotemporally-restricted manner in vivo, we show that CB1R located on corticostriatal projections, by blunting glutamatergic output, selectively safeguards D1R-MSNs of the mouse dorsal striatum from cortical mutant huntingtin (mtHtt)-induced damage. Specifically, expression of mtHtt in the motor cortex damages D1R-MSNs but not D2R-MSNs upon (i) pharmacological blockade of CB1R with rimonabant or (ii) conditional genetic deletion of CB1R in cortical principal neurons. This neurotoxic process is rescued by administration of the NMDA receptor antagonist MK-801. Likewise, a selective vulnerability of D1R-MSNs vs. D2R-MSNs is observed when corticostriatal glutamatergic projections are overactivated remotely by means of a DREADD pharmacogenetics approach and CB1R is pharmacologically blocked. CB1R located on corticostriatal projections, by inhibiting glutamatergic transmission, also protects MSNs from mtHtt-expressing striatal astroglia. Finally, astroglial MGL controls the availability of the 2-AG to ensure protection of MSNs.

The results obtained in this Thesis allow us to obtain the following **CONCLUSIONS**:

I. Striatal circuits can be "turned on" by acute Gq-protein signalling or "turned off" by sustained Gq-protein signalling. Specifically, sustained Gq-protein signalling inactivates MSNs of the dorsal striatum by an intracellular pathway that relies on JNK.

II. CB1R located on corticostriatal projections, mainly by blunting glutamatergic output, selectively safeguards D1R-MSNs of the dorsal striatum.

Collectively, these findings define the molecular mechanism and functional relevance of Gq-protein-driven signals in striatal circuits under normal and overactivated states, and define cortical CB1R as a key neurochemical player in dictating a dissimilar vulnerability of D2R-MSNs vs. D1R-MSNs. Altogether, they may contribute to understand the role of coordinated cannabinergic-glutamatergic signaling in the control of the direct and indirect corticostriatal pathways, and their dysregulation in basal ganglia disorders.

ABBREVIATIONS

2-AG: 2-Arachidonoylglycerol

ABHD: α/β-Hydrolase

AC: Adenylyl cyclase

AEA: *N*-arachidonoylethanolamine (anandamide)

AMPK: AMP-activated protein kinase

ATF-2: Activating transcription factor 2

BAC: Bacterial artificial chromosome

BDNF: Brain-derived neurotrophic factor

BG: Basal ganglia

CaM: Calmodulin

CaMK: Calmodulin-dependent kinase

cAMP: 3′,5′-Cyclic adenosine monophosphate

CaN: Calcineurin (Ca^{2+}-sensitive protein phosphatase 2B)

CB1R: Cannabinoid receptor, type 1

CB2R: Cannabinoid receptor, type 2

CNS: Central nervous system

COX: Cyclooxygenase

CREB: cAMP response element-binding protein

CRIP1a: Cannabinoid receptor-interacting protein 1a

Cx: Cortex

D1R: Dopamine receptor, type 1

D2R: Dopamine receptor, type 2

DARPP-32: Dopamine- and cAMP-regulated phosphoprotein of 32 kDa

DAG: Diacylgycerol

DAGL: Diacylgycerol lipase

DGK: Diacylglycerol kinase

DPE: Depolarization-induced potentiation of excitation

DREADD: Designer receptor exclusively activated by designer drug

DSE: Depolarization-induced suppression of excitation

DSI: Depolarization-induced suppression of inhibition

DYN: Dynorphin

eCB: Endocannabinoid

ENK: Enkephalin

Epac: Exchange protein directly activated by cAMP

ERK: Extracellular signal-regulated kinase

FAAH: Fatty acid amide hydrolase

FAN: Factor associated with neutral sphingomyelinase activation

GABA: γ-Aminobutyric acid

GIRK: G-protein-activated inwardly rectifying K^+ channel

GLT-1: Glutamate transporter 1

GPCR: G protein-coupled receptor

GPe: Globus pallidus, external segment

GPi: Globus pallidus, internal segment

GRK: GPCR kinase 2

GSK-3β: Glycogen synthase 3β

HCN: Hyperpolarization-activated cyclic nucleotide-gated channel

HD: Huntington's disease

Htt: Huntingtin

IL-2: Interleukin-2

IP3: Inositol 1,4,5-*tris*phosphate

IP3R: Inositol 1,4,5-*tris*phosphate receptor

IT: Intratelencephalic neuron

JNK: c-Jun *N*-terminal kinase

KO: Knock-out

LTD: Long-term depression

LTP: Long-term potentiation

mAChR: Muscarinic acetylcholine receptor

MAPK: Mitogen-activated protein kinase

MGL: Monoacylglycerol lipase

mGluR: Metabotropic glutamate receptor

MSN: Medium-sized spiny neurons

mtHtt: Mutant huntingtin

mTOR: Mammalian target of rapamycin

mTORC1: Mammalian target of rapamycin complex 1

NAE: *N*-acylethanolamine

NAPE: *N*-acylphosphatidylethanolamine

NFAT: Nuclear factor of activated T-cells

NMDA: *N*-methyl-D-aspartate

NMDAR: *N*-methyl-D-aspartate receptor

NO: Nitric oxide

PA: Phosphatidic acid

PD: Parkinson´s disease

PDE: Phosphodiesterase

PI3K: Phosphatidylinositol 3-kinase

PIP2: Phosphatidylinositol 4,5-*bis*phosphate

PKA: Protein kinase A

PKC: Protein kinase C

PKD: Protein kinase D

PLA2: Phospholipase A2

PLC: Phospholipase C

PLD: Phospholipase D

polyQ: Polyglutamine

PP1: Protein phosphatase 1

PP2A: Protein phosphatase 2A

PPAR: Peroxisome proliferator-activated receptors

PSD-95: Postsynaptic density protein of 95 kDa

PT: Pyramidal tract

RasGRP: Ras guanyl nucleotide-releasing protein

ROCK: Rho-associated protein kinase

SNc: Substantia nigra *pars compacta*

SNr: Substantia nigra *pars reticulata*

STN: Subthalamic nucleus

SP: Substance P

Str: Striatum

THC: Δ^9-Tetrahydrocannabinol

TNFα: Tumor necrosis factor-α

TRPV1: Transient receptor potential cation channel subfamily V member I

VGCC: Voltage-gated Ca^{2+} channel

WT: Wild-type

wtHtt: Wild-type huntingtin

YAC: Yeast artificial chromosome

INTRODUCTION

CORTICOSTRIATAL CIRCUITRY

The **corticostriatal circuitry** includes the functional loop between the **cortex** (**Cx**), **basal ganglia** (**BG**) and **thalamus**, and is an integrative centre for numerous cognitive processes and motor functions, such as action selection, motor coordination, sequential learning and habituation (Shepherd 2013).

The **striatum** is the main entry to the BG circuitry, and receives excitatory input from the cortex and the thalamus, as well as dense innervation from mesencefalic dopaminergic neurons, mainly from the substantia nigra *pars compacta* (SNc). The vast majority (~95%) of neurons within the striatum are GABAergic **medium spiny neurons** (**MSNs**), which are topographically connected to the cortical surface and project directly ("**direct pathway**") or indirectly ("**indirect pathway**"), through the external segment of the globus pallidus (GPe) and through the subthalamic nucleus (STN), to the basal ganglia output nuclei, including the internal segment of the globus pallidus (GPi) and the substantia nigra *pars reticulata* (SNr) (Grillner et al. 2005) (Figure 1).

MSNs received their name owing to its morphology, characterized by a soma of approximately 12-20 µm in diameter, from which around 7-10 dendrites moderately branched and densely covered with thorns radiate. They also have a collateral axon that projects within the striatum, in addition to their projecting axon (DiFiglia et al. 1976). These neurons are divided into two populations, represented in approximately the same proportion, according to their direct or indirect connection with the projection nuclei of the BG, and although physically intermingled and morphologically indistinguishable, they can be differentiated by some specific markers. Thus, the direct pathway is formed by MSNs expressing **dopamine D1 receptor** (**D1R-MSNs**), substance P (**SP**), and dynorphin, while the indirect pathway is composed of MSNs expressing **dopamine D2 receptor** (**D2R-MSNs**), adenosine A2A receptor (**A2AR**), and encephalin (**ENK**) (Gerfen & Young 1988; Bäckman et al. 2003) (Figure 1).

D2R-MSNs are more excitable than D1R-MSNs as, at similar current intensities they fire more action potentials (Kreitzer & Malenka 2007), and have smaller dendritic surface area and bigger axospinous asymmetric corticostriatal terminals (Lei et al. 2004). In addition, D2R-MSNs display a higher frequency of spontaneous excitatory postsynaptic currents and a unique pattern of large-amplitude excitatory events, all of which supports the idea that D2R-MSNs may be intrinsically more susceptible to damaging excitatory inputs (Kreitzer & Malenka 2008).

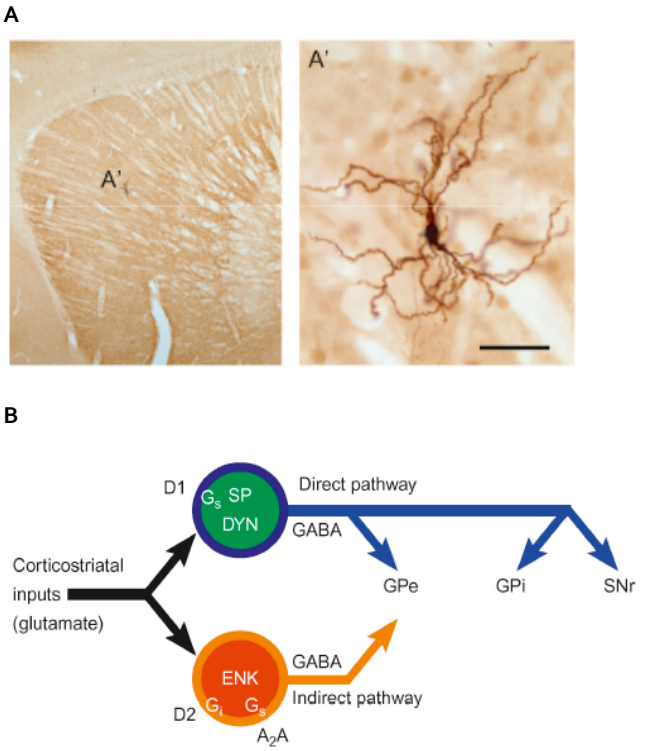

Figure 1. The striatal medium spiny projection neuron. A. Striatum. **A´**. High magnification of a medium spiny neuron. **B.** Diagram of the direct and indirect pathway neurons. Both neurons are GABAergic and receive glutamatergic corticostriatal inputs. Direct pathway neurons express the D1 receptor, the stimulatory Gs/olf G-proteins, as well as the neuropeptides substance P (SP) and dynorphin (DYN). These neurons project to the GPe, GPi and SNr. Indirect pathway neurons express the D2 receptor, the A2A adenosine receptor, and the neuropeptide ENK. The D2 receptor is coupled to the inhibitory Gi-protein, while the A2A receptor is coupled to the stimulatory Gs/olf G-protein. Adapted from Gerfen & Bolam 2010.

Much fewer in number, but not necessarily in functional relevance, are the **interneurons**, which account for the remaining 5% of striatal neurons. The striatum is essentially composed of one type of **large cholinergic** interneurons, and three types of neurochemically and electrophysiologically distinct GABAergic interneurons: **medium-sized GABA/parvalbumin** interneurons (also known as fast-spiking interneurons), **GABA/calretinin** interneurons, and **somatostatin/neuropeptide Y/nitric oxide synthase/ GABA** interneurons. GABAergic interneurons play a predominant role in regulating spike timing in the spiny output neurons through feedforward inhibition, while cholinergic interneurons discharge tonically. All striatal interneurons are aspiny in comparison to the striatal spiny projection neurons (Goldberg & Wilson 2010; Tepper 2010).

The three major types of **glial cells** present in the central nervous system (CNS), namely **astrocytes**, **oligodendrocytes**, and **microglia**, also reside within the striatum. All these types are key players for the functional fine tuning within the circuitry. Particularly, astrocytes have been shown to couple their activity to a specific subset of neurons belonging to the direct or

indirect pathway, and they subsequently respond attempting for balance between both networks (Martín et al. 2015).

Alterations at any level of the corticostriatal and BG circuitry can give rise to pathophysiological changes that are characteristic of many neurological diseases, including **Huntington´s disease** (**HD**), **Parkinson's disease** (**PD**), schizophrenia, obsessive-compulsive disorder, autistic spectrum disorder and other pathologies.

THE DIRECT AND INDIRECT PATHWAYS

The striatum act as an integrative node where MSN's information depends on cortical and thalamic inputs. Cortical neurons providing striatal inputs are located in several cortical areas including sensory, motor, and association regions. The connection is bilateral, with an ipsilateral predominance, and it is thought to provide the striatum with the sensory and motor planning information it needs to execute its role in motor control. The topographic organization of corticostriatal projections was embodied in the concept of functional regions within the striatum being dependent on the cortical origin of inputs to these regions (Alexander et al. 1986). Thus, dorsal regions of the striatum receiving inputs from premotor and motor cortical areas are characterized as "motor" regions of the striatum, whereas more ventral regions receiving inputs from limbic cortical areas are characterized as "limbic". **Corticostriatal neurons** are divided in two types of cortical excitatory neurons: **intratelencephalic** (**IT**) neurons, which project ipsilaterally or bilaterally (via the external capsule and corpus callosum) within the telencephalon (cortex and striatum) and can be found in layers II–VI of the cortex, and **pyramidal tract** (**PT**) **neurons**, which are restricted to deep layer V, projecting to the brainstem and in some cases also to the spinal cord (via the internal capsule, cerebral peduncle and pyramidal tract), and extend branches to the ipsilateral cortex and numerous subcortical regions including the striatum.(Shepherd 2013).

The **cortical excitatory input** to the striatum functions as a **dysinhibitory mechanism**. Thus, activation of D1R-MSNs by excitatory input from the cortex results in inhibition of the tonic inhibitory output of the basal ganglia, resulting in locomotor activation/movements. On the contrary, the main target of D2R-MSNs are GABAergic neurons in the GPe, which project to the output neurons of the basal ganglia and to the STN. Thus, cortical excitation of the indirect pathway inhibits the GPe, resulting in dysinhibition of the output neurons of the basal ganglia and the STN, thus decreasing movement (Gerfen & Bolam 2010) (Figure 2). This classical model, however, entails a simplification of one of the most hyper-connected areas within the CNS, and the specific functional outputs from interactions between each of its components are still unknown. Nonetheless, it is well accepted that, in general terms, the activity of the direct and indirect striatal pathways provides counterbalanced or antagonistic regulation of BG output, and this activity switch plays a central role in motor control and action initiation (Donahue & Kreitzer 2015).

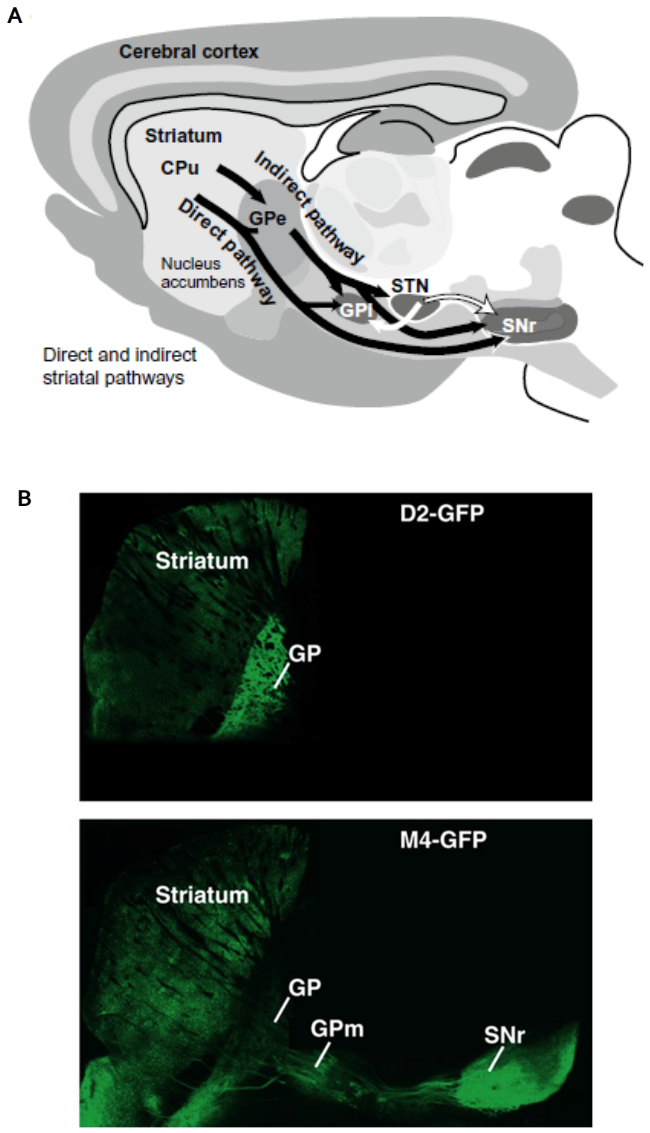

Figure 2 Direct and indirect pathways. A.The direct and indirect striatal projection pathways arise from D1R-MSNs and D2R-MSNs, respectively. D1R-MSNs, providing an axon with collaterals to the GPe, project to the GPi and SNr output nuclei. D2R-MSNs are indirectly connected to the GPi and SNr through connections that involve the GPe and the STN. B. Sagittal slices from a BAC transgenic mouse expressing GFP under the control of genomic regulatory elements for the dopamine D2 receptor (D2-GFP), or the muscarinic M4 receptor (M4-GFP), which labels indirect- and direct pathway MSNs, respectively. Adapted from Kreitzer & Malenka 2008; Gerfen &Bolam 2010.

To complicate things further, **dopaminergic neurons** projecting from SNc modulate the activity of both pathways by means of D1R-MSNs and D2R-MSNs.

Dopamine signalling trough D1R and D2Rhas opposite cellular consequences, since it engages stimulatory **Gαs/olf** or inhibitory **Gαi/o** proteins, respectively (Bronson & Konradi 2010; Surmeier et al. 2007), but in both cases it ultimately **enhances movement** (Albin et al. 1989). By modulating these two MSN populations, dopamine is believed to regulate not only ongoing choices about what to do and what not to do, but also to lay down a memory of the consequences of those choices that helps guide future behaviour (Freeze et al. 2013).

Most previous data on the two MSNs populations are based on the use of dopamine receptor knockout mice, pharmacology treatments and selective expression of toxins that generate cell-type-specific lesions or inactivation (Kreitzer & Berke 2011). The difficulty to distinguish between the two MSN populations has been largely overcome in the past years by the development of **BAC transgenic mice**, using either D1R or M4 lines to identify direct-pathway MSNs, and D2R or A2AR lines to identify indirect-pathway MSNs (Gong et al. 2007). Experiments based on these lines have revealed that D1R-MSNs and D2R-MSNs actually exhibit significant differences in dendritic morphology (Gertler et al. 2008), excitability (Kreitzer & Malenka 2007), gene expression (Gerfen & Young 1988), response to local GABAergic input, and mechanisms of plasticity (Kreitzer & Malenka 2007).

One example of such studies is the neuronal expression of **long-term depression** (**LTD**) and **long-term potentiation** (**LTP**) processes. MSNs show characteristic shifts of membrane potential between two preferred levels, one that is more polarized, so-called the down-state (varying from -94 to -61 mV), and another one that is more depolarized, so-called the up-state (varying from -71 to -40 mV), triggered by increased activity of many convergent corticostriatal neurons (Wilson & Kawaguchi 1996). Manipulation of excitability of MSNs, considering these two levels, can trigger striatal LTP or LTD. LTD has been shown to be D2R-dependent (Kreitzer & Malenka 2007) but, although still a matter of debate, instead of being a D2R-MSN-specific process, it seems to be present at both types of MSNs depending on the stimulation protocol used, and maybe involving a cholinergic interneuron-mediated mechanism in the case of D1R-MSNs (Wang et al. 2006; Bagetta et al. 2011). On the other hand, D1R has been implicated in striatal LTP (Di Filippo & Calabresi 2010). However, it has also been described that LTP could be elicited at both D1R-MSNs and D2R-MSNs, being dopamine-independent in D2R-MSNs, which required A2A receptors instead (Shen et al. 2008). Together, these distinct properties are believed to endow each MSN population with unique functions within the BG network.

In addition, **optogenetics** has provided a valuable tool for dissecting direct and indirect corticostriatal pathway functional output (Kreitzer & Berke 2011). This has unveiled that, according to the BG classical model, excitation of D1R and D2R-MSNs acts in a bidirectional way on **locomotion**. For example, it has been shown how bilateral excitation of indirect pathway MSNs elicited a parkinsonian-like state, characterized by freezing, bradykinesia and decreased locomotor initiation, while activation of direct pathway MSNs reduced freezing and increased locomotion (Kravitz et al. 2010) In addition, by manipulating direct and indirect striatal pathways, it was reported that both, **goal-directed learning**, which depends on reward outcome, and **habit formation**, are totally dependent on striatal function. In this context, activation of direct-pathway circuits has been proposed to facilitate or select appropriate movements, whereas activation of the indirect pathway may inhibit unwanted or inappropriate movements (Kreitzer & Malenka 2008). Interestly, recent data support that activation of both direct and indirect pathways occurs during action initiation (Cui et al. 2013). Actually, the two MSN populations are involved in a different way during sensorimotor processing and goal-directed behaviour (Sippy et al. 2015; Lee et al. 2016).

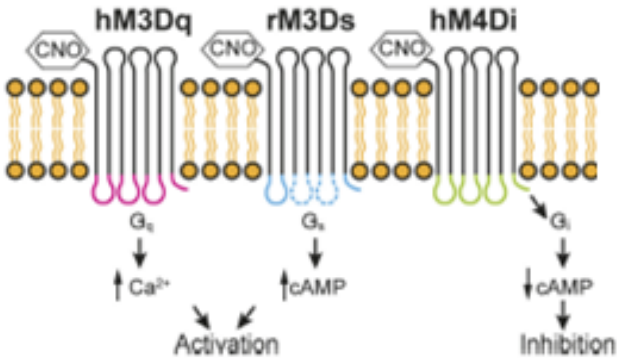

Figure. 3. Designer Receptors Exclusively Activated by Designer Drug (DREADDs) with their pharmacologically inert ligand clozapine-N-oxide (CNO) and G protein-coupling properties. hM3Dq and hM4Di are point-mutated human M3 and M4 muscarinic DREADDs, coupled to Gq and Gi signalling, respectively. rM3Ds consists of a chimeric point-mutated rat M3 muscarinic DREADD with intracellular loops from the turkey β1-adenoceptor, coupled to Gs signalling. CNO stimulation of hM3Dq and hM3Ds activates neurons. CNO stimulation of hM4Di inhibits neurons. Adapted from Sizemore et al. 2016.

Another recently developed tool, the **designer receptors exclusively activated by designer drugs** (**DREADDs**) (Figure 3), has been applied for assessing the remote control of neuronal activity (Rogan & Roth 2011). This chemogenetic method is based on the expression of engineered **G protein-coupled receptors** (**GPCRs**) that are selectively and potently activated by systemically bioavailable, brain-penetrant and otherwise pharmacologically inert

ligands such as clozapine-*N*-oxide (CNO) (Armbruster et al. 2007). DREADDs have no detectable constitutive activity and, by using conserved and canonical GPCR-dependent signalling pathways, allow a spatiotemporally-selective and physiological manipulation of them. Moreover, since G protein-coupled receptor pathways are involved in a multitude of CNS disorders, their manipulation both illuminates disease processes and identifies potential avenues of treatment.

The DREADD technology is broadly used in neurosciences (Wess et al. 2013). Specifically in the striatum, expression of **hM4Di** (Gi-coupled DREADD), the most commonly used inhibitory DREADD, drives synaptic plasticity (Kozorovitskiy et al. 2012), and modulated amphetamine sensitization in a MSN-type specific way (Ferguson et al. 2011). On the other hand, activation of MSNs through **hM3Ds** (Gs-coupled DREADD) has been also performed in the context of decision-making (Ferguson et al. 2013) and locomotor behaviour (Farrell et al. 2013). The first report that CNO-induced activation of **hM3Dq** depolarized and excited genetically defined neurons appeared in 2009 (Alexander et al. 2010). Since then, hM3Dq has been widely used to enhance neuronal firing, modulating processes as locomotion and striatal synaptogenesis (Kozorovitskiy et al. 2012). Because hM3Dq activation induces intracellular Ca^{2+} release, it has also been used to "activate" astrocytes (Bang et al. 2016), and iPS-derived neuroblasts (Dell'Anno et al. 2014) (Figure 3).

GPCRs AND STRIATAL CELL FUNCTION

Metabotropic signalling is absolutely necessary for the proper functioning of the striatum, and many neurotransmitters/neuromodulators such as dopamine, glutamate, adenosine, acetylcholine and endocannabinoids control the activity and plasticity of MSNs by engaging various members of the GPCR superfamily. **GPCRs** are integral membrane proteins that mediate signals from, for example, neurotransmitters, hormones, neuropeptides and cytokines. They can be divided into three families. Family 1 is the largest and contains the majority of GPCRs, including opioid, muscarinic, adenosine, cannabinoid, dopamine, somatostatin and tachykinin receptors, amongst others. Family 2 includes a number of peptide hormones and is not significantly represented in the BG. Family 3 is a small family but includes the metabotropic glutamate and GABA receptors, which are of considerable importance in the BG (Emson et al. 2010). All GPCRs contain seven transmembrane domains and derive their name from the interaction with intracellular **heterotrimeric G proteins**, **Gαβγ**. Upon activation by GPCRs, G proteins exchange GDP for GTP, causing dissociation of the Gα subunit from the Gβγ subunit, and subsequent initiation of a plethora of internal signalling pathways (Bronson & Konradi 2010) (Figure 4).There are four families of Gα subunits, three of them, **Gi/o**, **Gs/olf** and **Gq/11**, are majorly involved in BG receptor signalling. The Gi/o family is usually termed inhibitory as these G proteins inhibit adenylyl cyclases (**ACs**) (Figure 4) and are sensitive to pertussis toxin ribosylation, which prevents them from being aactivated by GPCRs. Apart from ACs, Gi also inhibits Ca^{2+} channels, activates mitogen-activated protein kinases (**MAPKs**) and can interact with other signalling pathways. Members of the Gs family, which includes Golf very abundantly in MSNs, all stimulate ACs (Figure 4), but can also activate **tyrosine kinases** (Gs) or **phosphoinositide turnover** (Golf). Members of the Gq/11 family stimulate, among several effectors, the various **phospholipase Cβ** (**PLCβ**) isoforms (Figure 4) (Bronson & Konradi 2010).

Among the different GPCR effectors, AC and its product, cyclic AMP (**cAMP**), are highly relevant in MSN function. D1R as well as A2AR upregulate cAMP production through Gαs/olf proteins. Such elevation on cAMP can triggers multiple signalling pathways, such as protein kinase A (**PKA)**, extracellular signal-regulated kinase (**ERK**) and cAMP response element-binding protein (**CREB)** (Girault, 2012; Cahill et al., 2014). Other striatal receptors, such a D2R, groups 2 and 3 of metabotropic glutamate receptors (**mGluRs**), opioid receptors, cannabinoid receptors (**CBRs**), and muscarinic acetylcholine M2/4 receptors (**mAChRs**), inhibit AC activity trough Gαi, opposing Gαs/olf action and promoting hyperpolarization of the plasma membrane by inhibiting **Na^+** and **Ca^{2+} channels**, while opening **K^+ channels** (Surmeier et al. 2007).

Gq-coupled receptors, such as metabotropic glutamate mGlu1/5 receptors (**mGluR1/5**) and muscarinic acetylcholine M1/3/5 receptors, are also very important in the control of MSN excitability, and an overactive Gq protein-driven signalling has been shown to occur in various models of BG-related diseases such as Huntington's disease and drug addiction (Conn et al. 2005; Kreitzer 2009; Cahill et al. 2014).. Gαq/11 activates **PLCβ**, which converts phosphatidylinositol-4,5-*bis*phosphate into diacylglycerol (**DAG**) and inositol 1,4,5-*tris*phosphate (**IP_3**). The most prominent target of DAG is the protein kinase C (**PKC**) family of Ser/Thr kinases (Figure 4). However, a number of alternative targets with PKC homology domains exist as well. These include protein kinase D (PKD), diacylglycerol kinase (DGK), Ras guanyl nucleotide-releasing protein (RasGRP), chimaerins, and mammalian uncoordinated13 (Munc13).

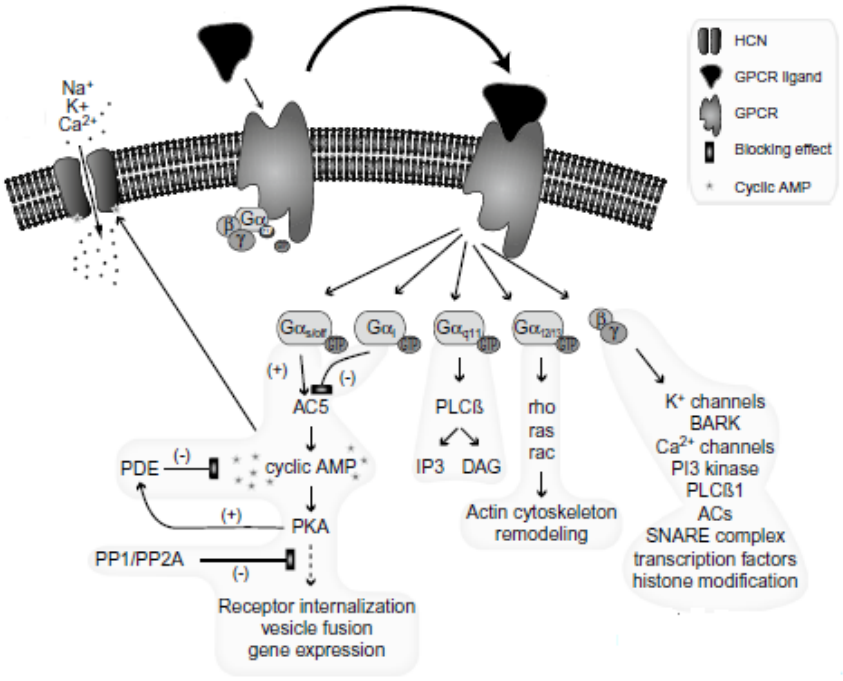

Figure 4. GPCR signalling pathways in the striatum. Scheme of the principal downstream effectors of GPCR activation in the striatum. After binding of the ligand to the GPCR, the Gβγ subunits dissociate from the Gα. Depending on the Gα subtype, different downstream pathways are activated. Gβγ subunits also trigger several cascade activation. Accumulation of cAMP can further stimulate HCNs and cation influx from extracellular space. PDE; phosphodiesterase, PP1/PP2A; protein phosphatase 1/ protein phosphatase 2, AC5; adenilato cyclase 5, HCN; hyperpolarization-activated cyclic nucleotide–gated channels. Adapted from Bronson & Konradi 2010.

IP_3, the second metabolite of phosphatidylinositol-4,5-*bis*phosphate hydrolysis, enhances Ca^{2+} release from the endoplasmic reticulum through its interaction with IP_3 receptors (**IP3R**) (Bronson & Konradi 2010).

The cytosolic rise in free **Ca^{2+}**, due to the influx from internal storages or from external sources upon activation of voltage-gated Ca^{2+} channels (**VGCCs**) or ligand-gated ion channels (such as NMDA and AMPA receptors), can activate a number of signal transduction pathways, including the cAMP/PKA pathway, the **Ca^{2+}/calmodulin** (**CaM**) / **CaM kinase** (**CaMK**) pathways, and the MAPK pathways. Known MAPK pathways include **ERK1** and **2**, c-Jun *N*-terminal kinase (**JNK**) **1, 2** and **3**, **p38 MAP kinases α, β, γ** and **δ**, and **ERK5.** MAPKs such as ERK1/2 can translocate to the nucleus, where they phosphorylate/activate downstream kinases and transcription factors such as **CREB**, **c-Jun**, **c-Fos** and others (Bronson & Konradi 2010).

The **Gβγ** dimeric subunit also participates in signal transduction pathways. First, it is required for Gα-mediated nucleotide exchange and for the inactivation of Gα subunits, but, in addition, it triggers various downstream cascades. Some examples, that have an important physiological role, include the inwardly-rectifying K^+ channels, GPCR kinase 2 (**GRK2**), **N-type** and **P/Q type Ca^{2+} channels**, phosphatidylinositol 3-kinase (**PI3K**), phospholipase A2 (**PLA2**), PLCβ, and ACs (Figure 4). On the presynaptic side, the Gβγ subunit can affect Ca^{2+} channel-mediated neurotransmitter release. It can directly bind to presynaptic Ca^{2+} channels and reduce the sensitivity to membrane depolarization Moreover, it has a direct inhibitory effect on the transmitter release machinery by binding to proteins of the SNARE complex. Finally, there is some indication that Gβγ might translocate to the cell nucleus, where it interacts with transcription factors and histone-modifying enzymes (Bronson & Konradi 2010).

Then, several molecules share common upstream activators, and the regulation of their activity can be triggered by different GPCR-related signalling pathways. For example, although activation of the ERK pathway in the striatum can be readily achieved by D1R and ionotropic (**NMDAR**) glutamate receptors, being a Gαs/olf-coupled protein and a Ca^{2+} permeable ion channel, respectively, mGluR1/5, which interact with Gq/11 proteins, also activates this pathway in synergy with D1R, thereby engaging, for example, drug-induced behavioural plasticity (Girault 2012).

In addition to interacting trough downstream signalling molecules, GPCRs in the striatum can also interact between each other, thus forming **heteromers**. One example is the **A2AR-D2R complex**, which couples to Gαq/11, thus opposing the activity of D2R alone. In that respect, it has been proposed that a GTPase-activating protein called regulator of G-protein signalling 4 (RGS4), links D2R and A2AR signalling to group I mGluR signalling, thus modulating endocannabinoid production by D2R-MSNs (Lerner & Kreitzer 2012). Furthermore, although it is well established that D1R and D2R are segregated in the adult striatum (Bäckman et al. 2003), in areas where they are co-expressed they can form hetero-oligomers that couple to Gαq/11 (Rashid et al. 2007). Very recently, heteromeric complexes of **A2AR-CB1R** have been fully characterized in the dorsal striatum. Coupling of these receptor heteromers to Gq proteins is found in D2R-MSNs, which leads to a decrease in the downstream signalling of both components, a process that becomes dysfunctional in HD (Moreno et al. 2017).

Hence, since there are multiple signalling possibilities inside one single striatal cell, integration of signalling properties from different G proteins plays a pivotal role in determining how the cell responds to multiple neurotransmitters that are active in BG. Imbalance in this fine modulatory network sets at the basis of several motor dysfunctions, and determines progression of BG neurodegenerative diseases.

STRIATAL CELL DISFUNCTION AND MOTOR DISORDERS

A large number of molecular and cellular mechanisms that lead to the degeneration of MSNs has been defined in preclinical models of striatal damage (Mitchell & Griffiths 2003; Han et al. 2010; Rikani et al. 2014). However, a still unsolved and intriguing aspect of neuronal death, not only in the corticostriatal circuitry but also in other brain areas affected by neurodegenerative diseases, is the identification of the precise molecular factors that determine the differential susceptibility of neuronal types.

Movement disorders, in general, result from or are coupled to an imbalanced activity of the direct and indirect striatal pathways. For example, in **PD**, which is marked by akinesia, the loss of dopaminergic input to the striatum, as a consequence of degeneration of the nigrostriatal dopamine system, has opposite effects on the direct and indirect pathways, with increased function of the indirect pathway and decreased function of the direct pathway (Gerfen 2010). As mentioned above, MSNs have some differences in morphology, molecular composition and electrophysiological properties and, therefore, it is conceivable that they have unequal responses against neurotoxic insults. For example, different signalling mechanisms in D1R-MSNs and D2R-MSNs occur in L-DOPA-induced dyskinesia, a major complication of the treatment of Parkinson's disease. which depends on both increased D1R–ERK signalling pathway in D1R-MSNs neurons and altered Ca^{2+} signalling in D2R-MSNs neurons (Valjent et al. 2009).

Particularly, in **HD**, in which the neurodegenerative process originates primarily from the cortex and striatum (Vonsattel & DiFiglia 1998), there is abundant evidence supporting that dissimilar alterations in the two MSN populations occur at different stages of disease progression. Over the years, a great number of HD models has been developed (Box 1). This, together with the emergence of highly efficient viral vectors for **mutant huntingtin** (**mtHtt**; the main protein linked to HD) delivery in the CNS, has thrown light on several aspects of corticostriatal dysfunction and clinical targets. Specifically, the viral vector approach offers some advantages over genetic mice models, as a local and massive overexpression of the disease gene, a rapid and highly flexible in vivo paradigm to study impact of mtHtt expression, and the possibility to avoid the emergence of compensatory mechanism by viral infection in adult animals. Notably, the implementation of viral vectors with cell-specific promoters is particularly suited for dissecting the contribution of each component, within the circuitry, to the pathogenic process (Ruiz & Déglon 2012).

Although **huntingtin** (**Htt**) is ubiquitously expressed, its mutant form (mtHtt) causes differential cell toxicity throughout the brain. Htt is a large protein of 348kDa that is subject to multiple transcriptional and post-transcriptional modifications, and it is involved in many cellular functions, from regulation of transcriptional activity to mitochondrial dynamics, vesicular trafficking or autophagy, to mention just a few. The pathogenic mechanisms of mtHtt have been largely described in the literature, and entail both loss and gain of protein function (Saudou & Humbert 2016). In addition to **cell-autonomous** effects, neurodegenerative progression in HD is also dependent on **non-cell-autonomous** processes. Among neuronal subtypes, the MSN receives the most massive combination of glutamatergic and dopaminergic input, which favours their increased vulnerability to damage. In fact, there are differential and complex imbalances in glutamate and dopamine modulation on D1R-MSNs and D2R-MSNs during HD progression (André et al. 2011).

BOX1. MODELLING HD

HD represents a prototypal model of a monogenic, fully penetrant neurodegenerative disorder characterized by an accumulation of misfolded proteins. Since the cloning of the *HTT* gene and identification of the mutation as a CAG expansion in the region that encodes the *N*-terminal part of the protein, a plethora of HD models have been developed.

CELLULAR MODELS

Cell lines offer a valuable tool for biochemical studies and are particularly suitable for transient, stable or inducible expression strategies. On the other hand, primary neurons or mixed cultures reproduce some cell–cell interactions, though not all the complexities of neuronal circuits, and these cultures have been extensively used to study disease pathogenesis and therapeutic screening. It can be mentioned a number of in vitro models:

1) **Neuron-like cell lines expressing mtHtt** with various length polyQ expansions (Lunkes & Mandel 1998).
2) **Primary neurons** derived from **transgenic HD mouse models** (Petersén A et al. 2001)
3) **Striatal cells** isolated from ***HTT* knock-in mice** (Trettel et al. 2000)
4) **Induced pluripotent stem cells** derived from **HD patients** (Park et al. 2008)

ANIMAL MODELS

In vivo models, although presenting differences among each other and none of them recapitulating completely human HD, have been the most powerful approach to characterize some of the key pathological features of the disease. Some examples are the following:

 # Fragment transgenic models

R6/2 mice (exon 1 around 150 CAG repeats)
Very aggressive, rapidly progressing phenotype, similar to the juvenile form of HD.
Overt behavioural symptoms as early as 4-5 weeks of age, die at about 15 weeks.
Alterations include nuclear inclusions (Davies et al. 1997), changes in neurotransmitter receptor expression (Cha et al. 1998; Ariano et al. 2002) and altered signalling mechanisms (Bibb et al. 2000; Luthi-Carter et al. 2000)
Motor (Carter et al. 1999) and learning deficits (Lione et al. 1999), as well as deficits in synaptic plasticity in the striatum of symptomatic animals (Raymond et al. 2011).
R6/1 mice (exon 1 around 110 CAG repeats)
Decreased mtHtt expression compared to R6/2 mice.
Similar phenotypic alterations as R6/2 but in a longer-term manner (Mangiarini et al. 1996).
Aberrant synaptic plasticity (Milnerwood et al. 2006).
ROSA-HD Cre/LoxP conditional mice (exon 1 with 103 CAG repeats)(Gu et al. 2005)
Cre dependent mtHtt expression in cortical pyramidal neurons and glia or all neural cells.
Cell-autonomous mHtt aggregation.
Progressive motor deficits and cortical neuropathology only observed when mtHtt expression is in multiple neuronal types.
GFAP-HD (208 aa of *N*-term with 106 polyQ) (Bradford et al. 2009)
Body weight loss, motor function deficits, and die earlier than wild-type mice.
mtHtt aggregation and gliosis, but not neuronal death.
Decreased expression of the glutamate transporter GLT-1.

 # Full-length transgenic models

Yeast artificial chromosome (**YAC**) expressing normal (YAC18) and mutant (YAC46, YAC72, and YAC128) full-length human Htt (Van Raamsdonk et al. 2007; Slow et al. 2003). They are named after the size of the polyQ repeat.
Bacterial artificial chromosome (**BACHD**) expressing full-length human mHtt with 97 polyQ (Gray et al. 2008). It is a conditional model, with loxP sites flanking *mtHTT* exon 1 with the polyQ repeat, and hence its expression can be genetically reduced in Cre-expressing cell lineages. Using this advantage it has been described, for example:
Reduction of cortical mtHtt expression improves striatal activity and behavioural deficits but does not improve neurodegeneration (Wang, Gray, X.-H. Lu, et al. 2014; Estrada-Sanchez et al. 2015).
Reduction of mtHtt expression in cortical and MSNs populations consistently ameliorates all behavioural deficits and selective brain atrophy (Wang, Gray, X.-H. Lu, et al. 2014).
Although with some differences, full-length models demonstrate slowly progressive but relatively robust motor dysfunction, psychiatric-related behaviours and cognitive deficits, and selective atrophy in the striatum and cortex (Lee et al. 2013).

 # Knock-in models

They express **full-length *mtHTT* in its native genomic context**. Several models differing mainly in the number of CAG repeats (from 48 to 200) have been generated (Levine et al. 1999; Trettel et al. 2000).They show behavioural abnormalities after one year of age, but present micro-aggregates very early in the course of the disease. Nuclear inclusions are observed only in older mice (10–18 months), and loss of MSNs occurs at about 2 years (Cepeda et al. 2010).

Classically, **D2R-MSNs** have been considered more vulnerable and the first to become dysfunctional in HD. Several lines of evidence from both HD genetic mouse models and postmortem brain samples from HD patients show a preferential loss of D2R-MSNs markers (Reiner et al. 1988; Ariano et al. 2002; Walker 2007). Disruption of D2R signalling, for example, is associated with alterations in the GSK3β pathway, triggering, for example, an impairment of the elimination of mtHtt aggregates through the ubiquitin-proteasome system (Rangel-Barajas & Rebec 2016b). In terms of motor symptoms, their preferential loss is believed to reduce the amount of inhibitory control over unwanted movements, leading to the chorea and hyperkinesia typically associated with HD (Walker, 2007).

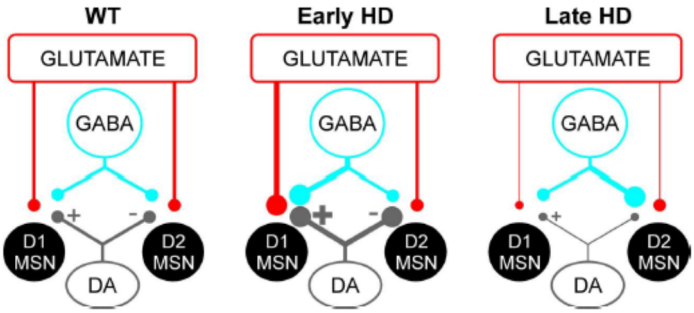

Figure 5. Representation of glutamate, GABA, and dopamine projections onto D1R-MSNs and D2R-MSNs in HD. In WT mice (left), DA released by nigrostriatal inputs activates glutamate and GABA release onto D1R-MSNs while it decreases glutamate and GABA release onto D2R-MSNs. In early HD (middle), increased DA transmission leads to increased release of glutamate and GABA onto D1R-MSNs. There is no change onto D2R-MSNs, suggesting that it might occur at different time points or involve other mechanisms. In late HD (right), D1R-MSNs display decreases in glutamate transmission, presumably due to loss of corticostriatal inputs and/ or decreased DA transmission. In contrast, D2R-MSNs display only a small decrease in glutamate synaptic inputs while GABA synaptic transmission is increased, probably through altered D2R function. Adapted from André, Cepeda, et al. 2011.

On the other hand, there is also data indicating that an early dysfunction of **D1R-MSNs** also occurs in HD patients (Hedreen & Folstein 1995), and genetic mouse models (André et al. 2011; Raymond et al. 2012). Thus, D1R signalling has been shown to be increased in pre-symptomatic HD mouse models, while its expression is decreased at symptomatic stages. The ERK signalling pathway, which is directly modulated by D1R, is altered in HD, and favours transcriptional dysregulation trough CREB, which, in turn, can accelerate the formation of mtHtt nuclear aggregates (Rangel-Barajas & Rebec 2016). These findings are also in line with a number of studies showing that D1R activation enhances NMDAR-evoked excitotoxic signalling on MSNs, while D2R activation usually reduces NMDA receptor-dependent responses (Cepeda & Levine 1998). In fact, D1R engagement has been shown to potentiate glutamate-evoked excitotoxic signalling to provoke the death of MSNs (Figure 5) (Cepeda & Levine 1998; Tang et al. 2007; Paoletti et al. 2008).

Additional evidence suggests that altered cellular and synaptic properties may result in aberrant overexcitation and eventual glutamate excitotoxicity (Levine et al. 1999). A particular point of view is that, although the loss of MSNs has an obvious role in the symptoms of HD, the onset and progression of the behavioural phenotype is rather caused by dysfunctional corticostriatal activity, which precedes cell death (Cepeda et al. 2007; Rangel-Barajas &. Rebec 2016). In fact, both cortical pyramidal neurons and MSNs show impaired electrophysiological properties along with HD-related motor deficits (Miller et al. 2008) Mice that model HD, for example, show behavioural symptoms long before significant cell loss. Moreover, these performance changes are accompanied by shifts in the intrinsic properties of MSNs. When studied in vitro, MSNs show exaggerated glutamate-dependent responses, more depolarized resting membrane potentials, reductions in both inwardly and outwardly rectifying K^+ currents, increased input resistance, and increased intracellular Ca^{2+} levels (Miller et al. 2008).

Changes in the glutamatergic system overlap with the functional alterations observed in **astrocytes** from HD patients and mouse models (Shin et al. 2005; Faideau et al. 2010; Jiang et al. 2016). Astrocytes are the main responsible cells for glutamate clearance from the synaptic cleft. In HD, their specific **glutamate transporter GLT-1** is downregulated at very early stages of the neurodegenerative process (Liévens et al. 2001; Jiang et al. 2016). In addition, recent work in an HD mouse model has shown that astrocytes downregulate the expression of a key K^+ channel (**Kir4.1**), resulting in an elevation in extracellular K^+ concentration. This rise in extracellular K^+ would depolarize MSNs and make them more vulnerable to NMDAR-evoked excitotoxicity (Tong et al. 2014).This strong exposure to glutamate excitotoxicity may be counteracted by some presynaptic receptors, located on corticostriatal terminals, such as group II metabotropic glutamate (**mGluR2** and **mGluR3**), $GABA_B$ (**$GABA_BR$**), cannabinoid (**CB1R**), and adenosine (**A1R**) receptors (Cepeda et al. 2010). Any functional alteration in cortical pyramidal neurons, or in the receptors on its corticostriatal terminals, can be critical for the impairment and neurodegeneration of the whole corticostriatal circuitry.

THE (ENDO)CANNABINOID SYSTEM

CANNABINOID RECEPTORS

The hemp plant (***Cannabis sativa* L.**) and its derivatives have been used in medicine for at least 50 centuries. However, the chemical structure of their active components (the cannabinoids) was not elucidated until the early 1960s (Gaoni & Mechoulam 1964), when the principal component of marihuana in terms of abundance and potency, **Δ9-tetrahydrocannabinol** (**THC**), was isolated from the plant. Around 30 years later, the first receptor of these molecules was cloned from rat cerebral cortex (Matsuda et al. 1990), human brain and testis (Gérard et al. 1991), and mouse brain (Chakrabarti et al. 1995), and termed as **cannabinoid receptor 1** (**CB1R**). Shortly after, a second cannabinoid receptor was identified in rat (Munro et al. 1993), and mouse (Shire et al. 1996), and named **cannabinoid receptor 2** (**CB2R**). Finally, the crystal structure of CB1R has been obtained very recently (Hua et al. 2016; Shao et al. 2016) (Figure 6), which opens a new and promising opportunity for understanding the biochemistry of these molecules.

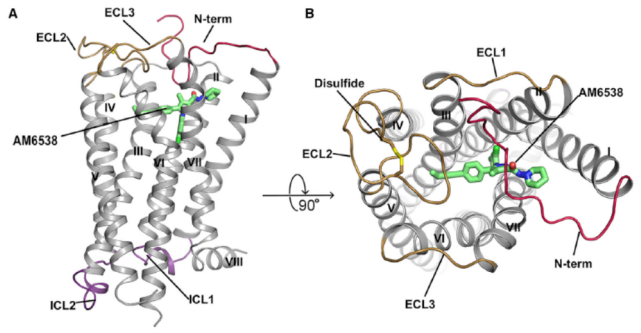

Figure 6. Crystal structure of the CB1R-AM6538 complex. **A** side view of the CB1R-AM6538 complex. The receptor is shown in grey cartoon representation. The ligand AM6538, shown with green sticks, demarcates the binding pocket, which is partially occluded by the *N*-terminal loop (red). The nitrate group is not modelled in the experimental crystal structure, as the electron density was not observed. The extracellular loops (ECLs) are shown in brown and the intracellular loops (ICLs) are shown in purple. **B** Top view of the extracellular side. The disulphide bond in ECL2 is shown as yellow sticks. Adapted from Hua et al. 2016.

There is a 44% homology between CB1R and CB2R, and they are encoded by different genes, giving rise to several isoforms of each receptor. They belong to the GPCR superfamily, and therefore are essentially composed of 7 transmembrane domains plus an *N*-terminal and a *C*-terminal domain. In addition, a third GPCR, named **GPR55**, with 14% of sequence similarity with CB1R and CB2R (Ross 2009), interacts specifically with some cannabinoid compounds and it has been shown to mediate part of their effects, at least *in vitro*, in a number of pharmacological studies (Ryberg et al. 2007; Kapur et al. 2009). Other protein sometimes considered a cannabinoid receptor, owing to its ability to bind some fatty acylethanolamides and *N*-arachidonoyldopamines, is the **transient receptor potential cation channel subfamily V member I** (**TRPV1**), a ligand-dependent ion channel responsible for the response to capsaicin (Bisogno et al. 2001; Pertwee et al. 2010). Lastly, some nuclear receptors of the **peroxisome proliferator-activated receptor** (**PPAR**) family could also respond to cannabinoids, at least in some contexts, although in a more subtle way and less sensitive manner than CB1R and CB2R (O'Sullivan 2007; Pertwee et al. 2010).

CB1R is the one of the most abundant GPCRs in the mammalian brain (Herkenham *et al.*, 1990; Kano *et al.*, 2009). It is preferentially expressed in areas of the CNS involved in the control of motor activity (**basal ganglia**, **cerebellum**), learning and memory (**cortex**, **hippocampus**), emotions (**amigdala**), sensory perception (**thalamus**), and various autonomic and endocrine functions as feeding behaviour, body temperature maintenance and pain perception (**hypothalamus**, **medulla**) (Figure 7). Indeed, CB1R is involved in critical life processes such as **nervous system development** and **inflammation** (Katona & Freund 2012; Lutz et al. 2015). CB1R is mainly present in mature **neurons**, and its expression is higher in **GABAergic** than **glutamatergic** populations (Marsicano & Lutz 1999). However, this expression pattern does not account for its activity potential, which reflects most likely the efficacy of receptor coupling to intracellular transductors (Steindel et al. 2013). In addition, it is expressed in other neural cells such as **astrocytes** (Sánchez et al. 1998; Stella 2004), where it modulates neuron-astrocyte communication (Venance et al. 1995; Navarrete & Araque 2008), **microglia** (Stella 2004; Stella 2010), and **oligodendrocytes** (Molina-Holgado et al. 2002).

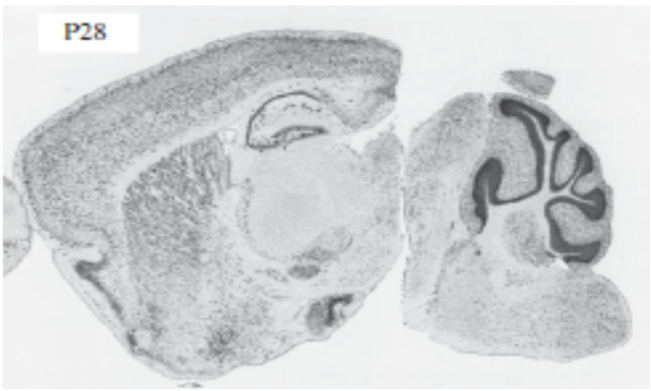

Figure 7. Expression pattern of CB1R mRNA in adult mice. CB1R mRNA *in situ* hybridization is shown at the indicated stage. Adapted from Diaz Alonso et al. 2012.

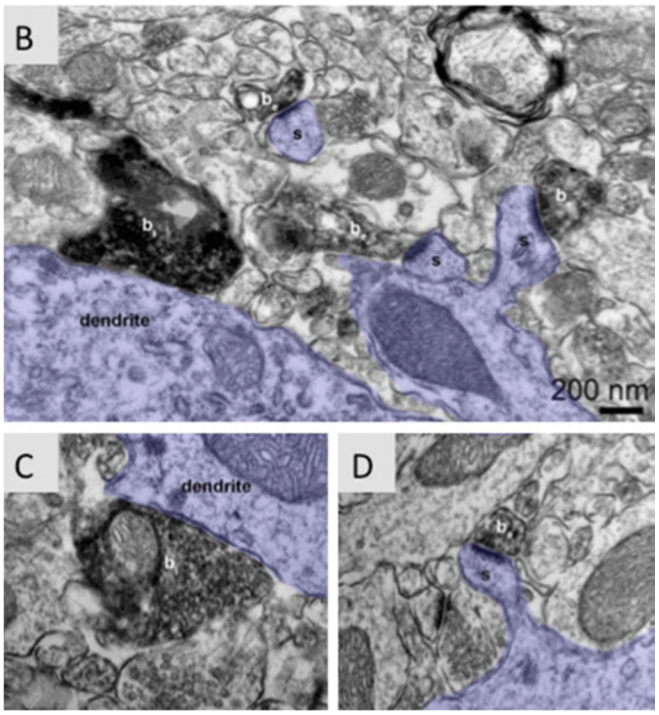

Figure 8. CB1R expression in rodent brain is primarily presynaptic. **B**. Electron-dense reaction product is present in both inhibitory (bi) and excitatory (be) boutons. Note that the excitatory boutons synapse onto spines (s) while the inhibitory boutons form synapses on a dendritic shaft. Dendritic structures are pseudocolored blue for ease of identification. **C**. Higher magnification image showing a CB1R-positive inhibitory terminal synapsing onto the shaft of a dendrite. **D**. Higher magnification image showing a CB1R-positive excitatory bouton forming an asymmetric synapse onto a dendritic spine. Scale bar ¼ 200 nm in B. Adapted from Hu & Mackie 2015.

At the cellular level, CB1R is mostly located at the **plasma membrane** of synaptic buttons, where it is well known that it inhibits neurotransmitter release (Figure 8) (Katona et al. 1999; Katona et al. 2006). However, it can also be found in the **postsynaptic zone**, participating in cell-autonomous regulation processes (Bacci et al. 2004; Marinelli et al. 2008; Maroso et al. 2016). Additionally, part of the receptor is present on intracellular structures, mostly **endosomes**, due to its constitutive recycling (Leterrier et al. 2004). A new location for CB1R, described in the last years, is the outer membrane of the **mitochondria**. There, cannabinoid compounds are able to modulate respiration, and therefore neuronal activity, thereby interfering with memory processes (Bénard et al. 2012; Hebert-Chatelain et al. 2016).

CB2R is preferentially expressed in the periphery, mainly in cells (B and T lymphocytes, macrophages) and tissues (spleen, lymph nodes) of the **immune system** (Herkenham et al. 1990; Glass et al. 1997; Tsou et al. 1998), **hematopoietic system** (Cabral & Staab 2005), **endocrine pancreas** (Juan-Picó et al. 2006), **bone** (Idris et al. 2005) and **adipose tissue** (Pagano et al. 2008). It is also present in the brain, mainly in **microglial cells** and, perhaps in some contexts, **astrocytes** (Stella 2004; Maresz et al. 2005). It has been also described that CB2 expression may occur in some restricted populations of **neurons**, although at low levels (Van Sickle et al. 2005; Lanciego et al. 2011; García et al. 2015), as well as in **neural progenitors** (Palazuelos et al. 2006).

THE ENDOCANNABINOID SYSTEM

Cannabinoid receptors interact with endogenous molecules known as **endocannabinoids** (**eCBs**). They present molecular similarities with the active ingredients of *C. Sativa*, the **phytocannabinoids**, thus enabling a functional mimesis between them (Pertwee et al., 2010). ***N*-arachidonoyletanolamine** or **anandamide** (**AEA**) was the first eCB identified (Devane et al. 1992), followed by **2-arachidonylglycerol** (**2-AG**) (Mechoulam et al. 1995; Sugiura et al. 1995).

Although there may be other endogenous molecules capable of interacting with cannabinoid receptors, AEA and 2-AG are to date the best established eCBs and the most relevant in terms of biological functions. 2-AG has lower affinity for cannabinoid receptors than AEA, but is a fully effective agonist at both CB1R and CB2R, and it is increasingly more accepted that it represents the main ligand of presynaptic CB1R in the control of synaptic activity and plasticity (Katona & Freund 2008). AEA is a high-affinity, low-efficacy CB1R agonist, with even lower efficacy at CB2R, and although it is also a presynaptic CB1R ligand, is likely more involved in complementary forms of eCB-mediated plasticity, for example though TRPV1 (Grueter et al. 2010; Puente et al. 2011).

Unlike most other neurotransmitters and neuromodulators, eCB generation occurs by "on-demand" synthesis and cleavage of plasma membrane lipid precursors, which are hydrophobic molecules with polyunsaturated fatty acyl chains, in a tightly controlled process related to neuronal activity (Mechoulam et al. 1998; Piomelli 2003). The principal pathway for **AEA** synthesis is from *N*-acylphosphatidylethanolamine (**NAPE**), which is in turn generated by *N*-acylation of phosphatidylethanolamine trough a Ca^{2+}-dependent *N*-acyltransferase. Subsequently, *N*-acylethanolamine (**NAE**) is released from NAPE by a phosphodiesterase of the phospholipase D type (**NAPE-PLD**), to finally generate phosphatidic acid (**PA**) and AEA (Di Marzo et al. 1994) (Figure 8). AEA synthesis is usually dependent on cytosolic Ca^{2+} elevation, but can also be Ca^{2+}-independent (Di Marzo et al. 1994; Leung et al. 2006). Furthermore, the activation of some GPCRs, like D2R, mGluR1/5R and M3R, can trigger AEA production (Piomelli 2003). **2-AG** is generated mainly from the

hydrolysis of phosphatidylinositol, first by **PLC**, and then by diacylglycerol lipase (**DAGL**). DAGL has two known isozymes, **DAGLα** and **DAGLβ** (Bisogno et al. 2003), being the α isoform the more abundant and apparently relevant within the adult brain (Gao et al. 2010; Tanimura et al. 2010). Neuronal 2-AG production can be initiated by an increase in cytosolic Ca^{2+} (Piomelli et al. 1997) triggered by the activation of metabotropic (Jung et al. 2007) or ionotropic (Stella & Piomelli 2001) receptors (Figure 9).

Degradation of eCBs, and therefore termination of their activity, is a fine-tune process, triggered by specific serine hydrolases. **Fatty acid amide hydrolase** (**FAAH**) is the main enzyme responsible for the degradation of AEA (Cravatt et al. 1996). and within the mammalian brain it is particularly highly expressed in the cerebellum, hippocampus and cortex (Leishman et al. 2016). **Monoacylglycerol lipase** (**MGL**) preferentially catabolizes 2-AG into arachidonic acid and glycerol and is widely expressed in the brain throughout the rat cortex, hippocampus, cerebellum, thalamus, striatum, amygdala and pontine nuclei (Dinh et al. 2002). These two hydrolases are differentially distributed across the synaptic cleft, being FAAH in the postsynaptic dendrites and soma while MGL resides in the presynaptic axon membrane, where it co-localizes with CB1R (Gulyas et al. 2004) (Figure 9).

Aside from these two major degradative pathways, both AEA and 2-AG can be metabolized by alternative ways. For example, the serine hydrolases **ABHD6** and **ABHD12** (Blankman et al. 2007; Marrs et al. 2010), and oxidative enzymes as **ciclooxygenases** and **lipoxygenases**, use these same eicosanoid substrates to generate different active metabolites (Nomura et al. 2011). In addition, eCB-degradation products are themselves precursors of bioactive lipid mediators, such as **prostaglandins**, **prostamides** and other **eicosanoids**, thus making their metabolic regulation and function tightly related, even more specially in particular cellular contexts such as an inflammatory response (Valdeolivas et al. 2013; Hermanson et al. 2014).

The location of enzymes engaged in the synthesis and degradation of eCBs is closely linked to the purpose of eCB signalling. As an example, DAGLα is found on the plasma membrane of the perisynaptic domain of neuronal soma, where is functionally coupled to **PLCβ** and **mGluR1/5** (Yoshida et al. 2006; Jung et al. 2007), and therefore it can modulate synaptic activity trough 2-AG synthesis and retrograde activation of presynaptic CB1R (Figure 11).

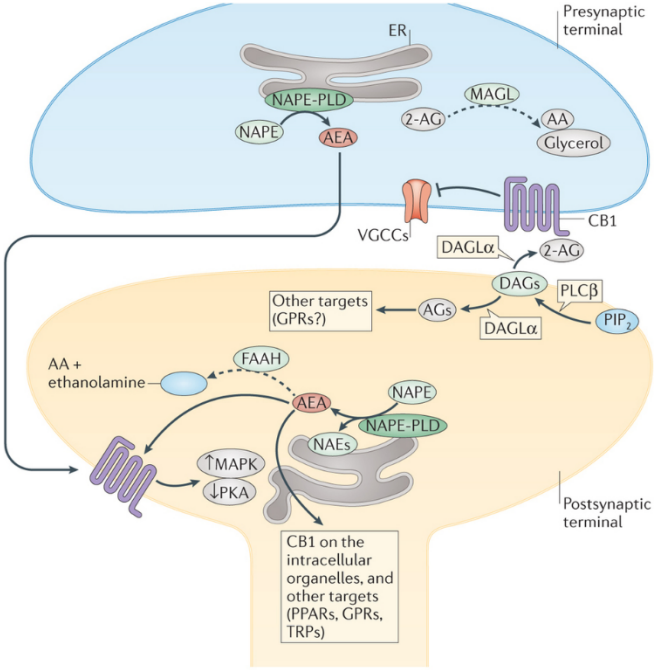

Figure 9. Main pathways for the synthesis and degradation of the endocannabinoids AEA and 2-AG. The subcellular distribution in neurons of enzymes regulating the levels of eCBs is shown, including the proposed role of these lipid mediators in retrograde (mainly for 2-arachidonoyl-glycerol (2-AG)), anterograde and intracellular (for anandamide (AEA)) signalling. The biosynthesis of AEA occurs through the action, among others, of NAPE-PLD, which is located in intracellular membranes both pre- and postsynaptically. AEA is degraded by FAAH, which is located postsynaptically. 2-AG is biosynthesized by DAGLα, which is located postsynaptically, and degraded by MGL, which instead is presynaptic. The complexity arising from the fact that many of these enzymes also regulate the levels of eCB-related mediators, with non-cannabinoid receptors as targets, is also depicted. Solid arrows denote transformation into active metabolites or activation; dashed arrows denote transformation into metabolites inactive at cannabinoid receptors; blunt arrow denotes inhibition. AA, arachidonic acid; AGs, 2-acylglycerols; DAGs, diacylglycerols; ER, endoplasmic reticulum; GPRs, orphan G-protein-coupled receptors; MAPK, mitogen-activated protein kinases; PIP2, phosphoinositide bisphosphate; PKA, protein kinase A; PLCβ, phospholipase Cβ; PPARs, peroxisome proliferator-activated receptors; TRPs, transient receptor potential channels; VGCCs, voltage-gated calcium channels. Adapted from Di Marzo et al. 2015.

CB1R SIGNALLING

Activation of cannabinoid receptors has multiple cellular consequences, and depends on the ligand as well as on the receptor-ligand engagement context (Glass & Northup 1999; Laprairie et al. 2017) Different scenarios could be given by the interaction of the receptors with other molecules, as other receptors with which they form **heteromers** (Mackie 2005; Ferré et al. 2010), or cytoplasmic interactors as **CRIP1a** (Niehaus et al. 2007), as well as **biochemical modifications** of the receptor (Shim 2010) .

Due to its GPCR nature, activation of CB1R or CB2R triggers GTP hydrolisis on a heteromeric G protein associated to it. Although interactions with other G proteins as **Gq/11** (Lauckner et al. 2005), **Gs** (Hampson

et al. 2000), or **G12/13** (Roland et al. 2014) has been shown to occur in particular contexts, the canonical signalling pathway involves **Gi/o** activation and the subsequent dissociation into the respective αi/o and βγ subunits (Pertwee 1997). Activation of the αi/o subunit leads to inhibition of **AC**, decrease in cAMP concentration, and hence reduction in the activity of cAMP-dependent cytoplasmatic proteins, such as PKA (Howlett 2002) and the exchange protein directly activated by cAMP (**Epac**) (Ramírez-Franco et al. 2014). On the other hand, the βγ dimers generated are related to different signalling pathways, for example **ERK** (Bouaboula et al. 1995; Galve-Roperh et al. 2002), **p38 MAPK** (Liu et al. 2000; Rueda et al. 2000), and **JNK** (Rueda et al. 2000; Derkinderen et al. 2001) (Figure 10).

Cannabinoids also regulate other pathways involved in the control of cell proliferation and survival, including the **PI3K-Akt pathway** (Gómez del Pulgar et al. 2000), **ceramide biosynthesis** (Galve Roperh et al. 2000), sphingomyelin hydrolysis through the adaptor protein **FAN** (Sánchez et al. 2001) and various **ion channels** (Pertwee 2005). For example, the well-known mechanism of CB1R-dependent inhibition of neurotransmitter release (Kreitzer & Regehr 2001) usually involves inhibition of N- and P/Q-type voltage sensitive Ca^{2+} channels (Caulfield & Brown 1992; Gebremedhin et al. 1999) and the activation of G protein-activated inwardly-rectifying K^+ (**GIRK**) channels (Guo & Ikeda 2004; Mackie 2005), which hyperpolarizes and thus reduces the excitability of the plasma membrane (Howlett 2002) (Figure 11).

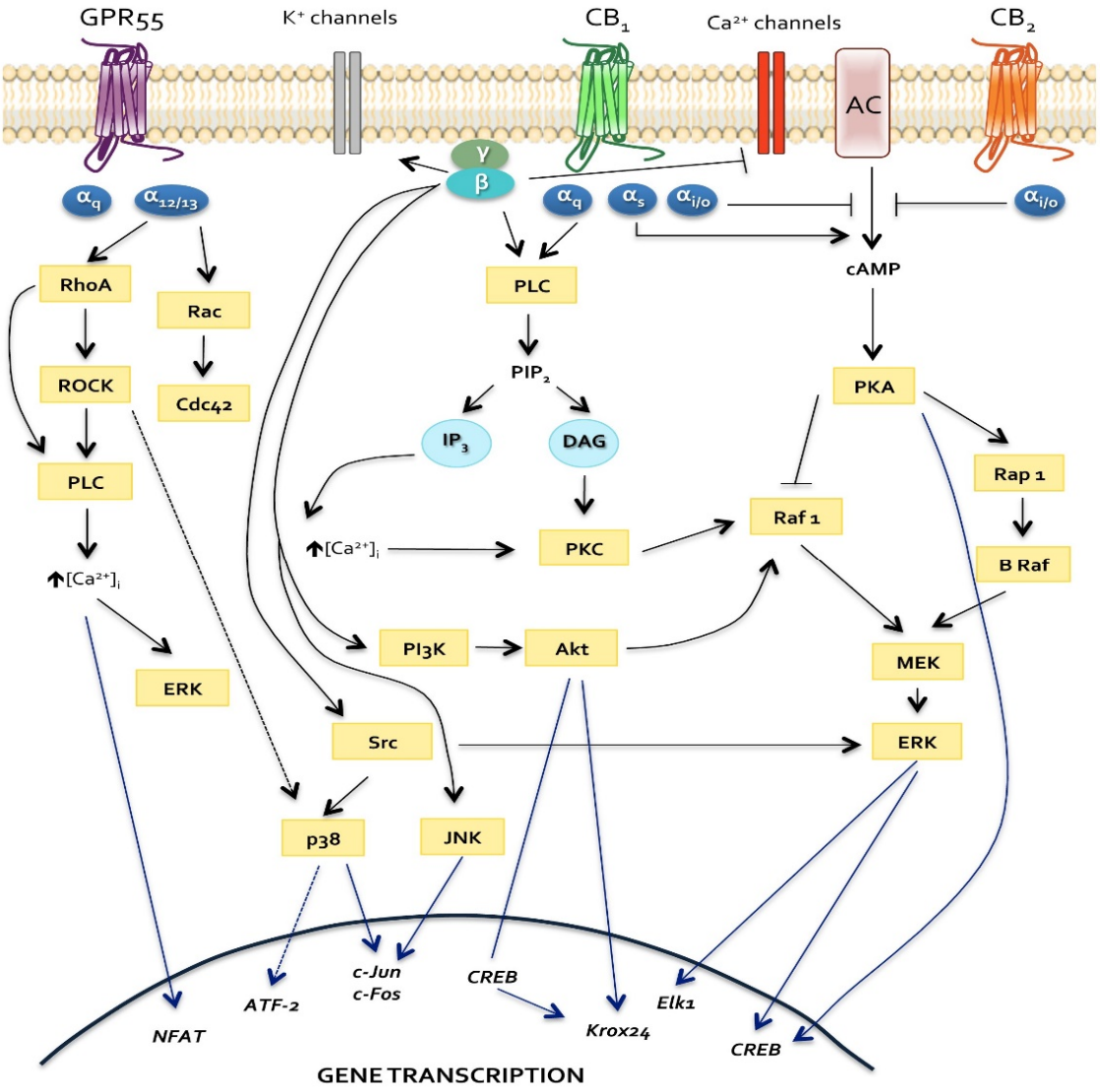

Figure 10. Cannabinoid receptors-coupled signalling pathways. The stimulation of cannabinoid receptors activates numerous signal transduction pathways, in a cell- and tissue-specific manner. AC, adenylyl cyclase; ATF-2, activating transcription factor 2; PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-*bis*phosphate; IP_3, inositol 1,4,5-*tris*phosphate; DAG, diacylglycerol; PKA, protein kinase A; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; ROCK, Rho-associated protein kinase; NFAT, nuclear factor of activated T cells; CREB, cAMP response element binding protein. Adapted from (André & Gonthier 2010)

Prolonged exposure to receptor agonists results in a decreased ability of CB1R and CB2R to further activate effector pathways, namely receptor **desensitization,** and in a reduction in the number of receptor molecules on the cell surface, namely receptor **internalization** (Dudok et al. 2014; Lu & MacKie 2016). Under these conditions, cannabinoid receptors become a substrate for GPCR kinases (**GRKs**), which phosphorylates serine and/or threonine residues on the cytoplasmic domains, thus enabling the interaction with **β-arrestins**. Binding of β-arrestins uncouples the receptor from G proteins and hence stimulates receptor internalization and β-arrestin-mediated signalling (Delgado-Peraza et al. 2016; Kendall & Yudowski 2017).

CB1R FUNCTIONS IN THE CNS

The eCB system plays a role in multiple physiological processes, both in the CNS and in peripheral tissues, in a cell- and tissue-specific manner. Due to the complexity of this system, which serves as a homeostatic modulator of several vital functions, it has become increasingly necessary to study, in a more in-detailed examination, the mechanism of action of its components. Moreover, eCBs offer a plethora of molecular targets and possible strategies to counteract multiple pathologies. Because the amount of physiological implications of cannabinoids exceeds by far the subject of this thesis, the following section focuses mostly on the role of CB1R in synaptic transmission and corticostriatal function. Finally, some of the protective aspects of cannabinoids in neurodegenerative diseases, with special emphasis on the role of CB1R, are also summarized.

CB1R and synaptic transmission

The best characterized mechanism by which eCBs modulate synaptic transmission trough CB1R involves a paracrine communication known as **retrograde signalling**. This term comes from the idea that, in contrast to the classical synaptic transmission, which is directed from the presynaptic to the postsynaptic zone, eCBs signal from the postsynaptic membrane, where they are generated, to the presynaptic terminal, where CB1R is located. The final consequence of this process is a reduction in neurotransmitter release, in a transient or long-term manner, on both excitatory and inhibitory synapses (Kano et al. 2009; Katona & Freund 2012).

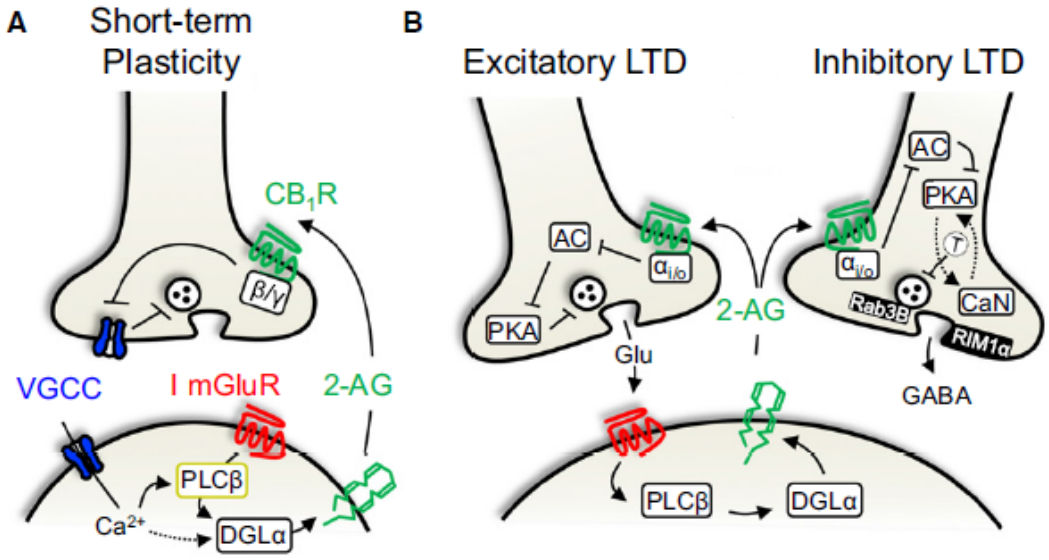

Figure 11 eCB-mediated short- and long-term synaptic plasticity. A Short-term depression. Postsynaptic activity triggers Ca^{2+} influx via VGCCs. Other Ca^{2+} sources, like NMDARs and internal stores, may also contribute. Ca^{2+} promotes DGLα-mediated eCB production by a still unclear mechanism. Presynaptic activity can also lead to eCB mobilization by activating postsynaptic group I metabotropic glutamate receptors (I mGluRs). PLCβ can now act as a coincidence detector integrating pre- and postsynaptic activity. DGLα promotes 2-AG release which retrogradely targets presynaptic CB1Rs, and the βγ subunits probably couple to presynaptic VGCCs to reduce neurotransmitter release **B** eCB-mediated excitatory long-term depression (LTD) and inhibitory LTD (iLTD). Patterned presynaptic stimulation releases glutamate (Glu) which activates postsynaptic mGluRs coupled to PLCβ and DGLα. 2-AG homosynaptically targets CB1Rs localized to excitatory terminals and heterosynaptically engages CB1Rs at inhibitory terminals. A Gαi/o-dependent reduction in AC and PKA activity suppresses transmitter release. At inhibitory synapses, decreased PKA activity, in conjunction with activation of the Ca^{2+}-sensitive phosphatase calcineurin (CaN), shifts the phosphorylation status of an unidentified presynaptic target (T) required for iLTD. The active zone protein RIM1α and the vesicle- associated protein Rab3B are also necessary for iLTD. Induction of eCB- LTD may require presynaptic Ca^{2+} rise through VGCCs, NMDARs, or internal stores (not shown). Dashed lines indicate putative pathways. Adapted from Castillo et al. 2012.

The first evidence for eCB-mediated retrograde signalling came from the study of a particular form of synaptic plasticity in which these molecules play an important role. This **short-term synaptic plasticity** is called depolarization-induced suppression of inhibition (**DSI**), in the case of inhibitory synapses (Ohno-Shosaku et al. 2001; Wilson & Nicoll 2001), or depolarization-induced suppression of excitation (**DSE**), in the case of excitatory synapses (Kreitzer & Regehr 2001). Soon after, it was found that eCBs can also mediate **long-term depression** events at the presynaptic level (**eCB-LTD**), in both excitatory (Gerdeman et al. 2002; Robbe et al. 2002) and inhibitory terminals (Marsicano et al. 2002; Chevaleyre & Castillo 2003) (Figure 11).

CB1R triggers the inhibition of neurotransmitter release by means of two major processes. In the case of short-term plasticity, it entails the inhibition of presynaptic Ca^{2+} influx trough VGCCs, most likely via the β/γ subunit of the Gi/o protein coupled to CB1R (Kreitzer & Regehr 2001; Wilson & Nicoll 2001; Brown et al. 2003). For long-term plasticity, the predominant mechanism requires inhibition of AC and downregulation of the cAMP/ PKA pathway via the Gαi/o subunit (Castillo et al. 2012). Induction of this form of plasticity requires combined presynaptic firing with CB1R activation, and thereby only active synapses detecting eCBs express long-term plasticity. The occurrence of eCB-LTD may involve the presynaptic proteins Rab3B/RIM1α (Tsetsenis et al. 2011; Castillo et al. 2012) (Figure 11).

The two main endocannabinoids, AEA and 2-AG, can modulate synaptic function. Both DSI and DSE requires 2-AG synthesis by DAGLα (Gao et al. 2010; Tanimura et al. 2010). On the other hand, AEA can participate in LTD, although at a slower rate than 2-AG, by acting both through CB1R (eCB-LTD), and through TRPV1 (**AEA-TRPV1-LTD**) in an autocrine manner, in which AEA activates the postsynaptic vanilloid receptor. AEA-TRPV1-LTD is present at both glutamatergic and GABAergic synapses (Chávez et al. 2010; Ohno-Shosaku & Kano 2014; Puente et al. 2015). There is a functional crosstalk between 2-AG and AEA signalling (Maccarrone et al. 2008), and some interesting findings suggest that 2-AG and AEA can be recruited differentially from the same postsynaptic neuron, depending on the type of presynaptic activity (Puente et al. 2011; Lerner & Kreitzer 2012).

In addition, arachidonic acid (**AA**), which is both a precursor (in a lipid-esterified or amidated form) and a degradation product of eCBs, has been recently found to act also as a retrograde messenger, thus potentiating excitatory transmission in a process called depolarization-induced potentiation of excitation (**DPE**). DPE has to be taken into consideration, as the metabolic balance of eCBs can lead to considerable changes in AA levels, thereby presumably influencing as well synaptic transmission (Carta et al. 2014; Lutz et al. 2015). Moreover, despite 2-AG and AEA being mainly hydrolyzed by MGL and FAAH, cyclooxygenase and lipoxygenase can also utilize these substrates to generate eCB-derived metabolites that are biologically active (Nomura et al. 2011) and conceivably modulate synaptic function (Yang & Chen 2008; R. Chen et al. 2013).

The eCB system in general, and CB1R in particular, are also present and functional in **astrocytes**. Thus, eCBs signalling is integrated into the concept of the '**tripartite synapse'**, including pre- and postsynaptic elements and surrounding astroglial processes (Navarrete & Araque 2008). Astrocytic CB1Rs couples to PLC via Gq/11, which increases intracellular Ca^{2+} and triggers glutamate release (Navarrete & Araque 2010). This form of plasticity implies an additional control level within neuronal circuits as, through astroglial networks, eCB signalling can modify, in a regulated and specific manner, synaptic activity of physically distant neurons (Navarrete & Araque 2010; Martín et al. 2015).

There are multiple physiological processes and behavioural aspects regulated by CB1R. To mention some examples, this receptor participates in the control of **extinction of aversive memories** and in **stress responses** (Marsicano et al. 2002; Senst & Bains 2014), **motor behaviour** (Herkenham et al. 1991; Katona & Freund 2008), **nocicepción** in both CNS and peripheral nervous system (Cravatt & Lichtman 2004), **energy balance** (Quarta et al. 2010), and **feeding behaviour** (Bellocchio et al. 2010; Soria-Gómez et al. 2014)**.** eCBs are central players in the maintenance of nervous system homeostasis. In this context, both **brain aging** and **neurodegenerative disorders** are associated to drastic changes in the eCBs system (Galve-Roperh et al. 2008; Fernández-Ruiz et al. 2010; Scotter et al. 2010; Di Marzo et al. 2015). In fact, eCBs safeguard the nervous system against **excitotoxicity**, **inflammation** and **oxidative stress**, thus acting as a counterbalance in neuronal overactivation contexts and constituting a brake againts neuronal death (Monory et al. 2006; Katona & Freund 2008).

CB1R in the corticostriatal circuitry

CB1R mRNA is particularly highly expressed within the **striatum** (**caudate-putamen)**, in a dorsolateral to ventromedial gradient (Figure 12) (Mailleux & Vanderhaeghen 1992; Marsicano & Lutz 1999), while adult pallidal and nigral neurons contain little or no

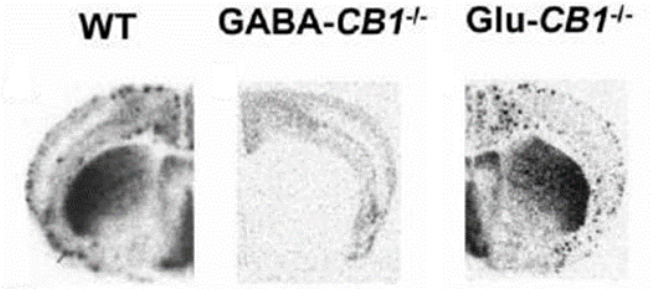

Figure 12. Expression pattern of CB1R in the cortex and striatum of conditional CB1R mutant mice. In situ hybridization showing CB1R mRNA expression in the brain of $CB1R^{f/f}$ (WT), Glu-$CB1R^{-/-}$, and GABA-$CB1R^{-/-}$ mice. In Glu-$CB1R^{-/-}$ mice, CB1R mRNA is absent from the majority of cortical glutamatergic neurons. In GABA-$CB1R^{-/-}$ mice, CB1R mRNA is absent from all GABAergic neurons and it is expressed only in non-GABAergic cells. Modified from Monory et al. 2006.

CB1 mRNA.(Matsuda et al. 1993). It is present at terminals of both D1R-MSNs and D2R-MSNs, where it mediates endocannabinoid-dependent inhibition of GABA release and thus reduction of motor activity (Herkenham et al. 1991; Katona & Freund 2008) (Fig. 13). In addition, CB1R is present at **cortical projection neurons**, but is not significantly present at other glutamatergic areas projecting to the dorsal striatum (Figure 13) (Mailleux & Vanderhaeghen 1992; Marsicano & Lutz 1999; Uchigashima et al. 2007; Wu et al. 2015). CB1R located on corticostriatal terminals thereby blunts glutamatergic output and gives rise to LTD (Gerdeman et al. 2002; Kreitzer 2009).

Signalling through CB1R is also linked to corticostriatal function in several contexts like reward and addiction (Di Marzo et al. 2001; Hansson et al. 2007; Lutz et al. 2015) as well as habit formation (Hilário et al. 2007; Gremel et al. 2016), mostly through the control of LTD and LTP. The eCB system modulates the activity of the neurotransmitters that participate in the control of basal ganglia function, namely dopamine, GABA and glutamate, to balance motor outputs. Since its location is mainly presynaptic (Figure 9), CB1R mediates corticostriatal plasticity by means of retrograde signalling (Figure 11) (Castillo et al. 2012), and its activation depresses corticostriatal glutamatergic synaptic transmission, thus being involved in the hypomotility and cataleptic effect induced by cannabinoid injection (Gough & Olley 1978; Monory et al. 2007).

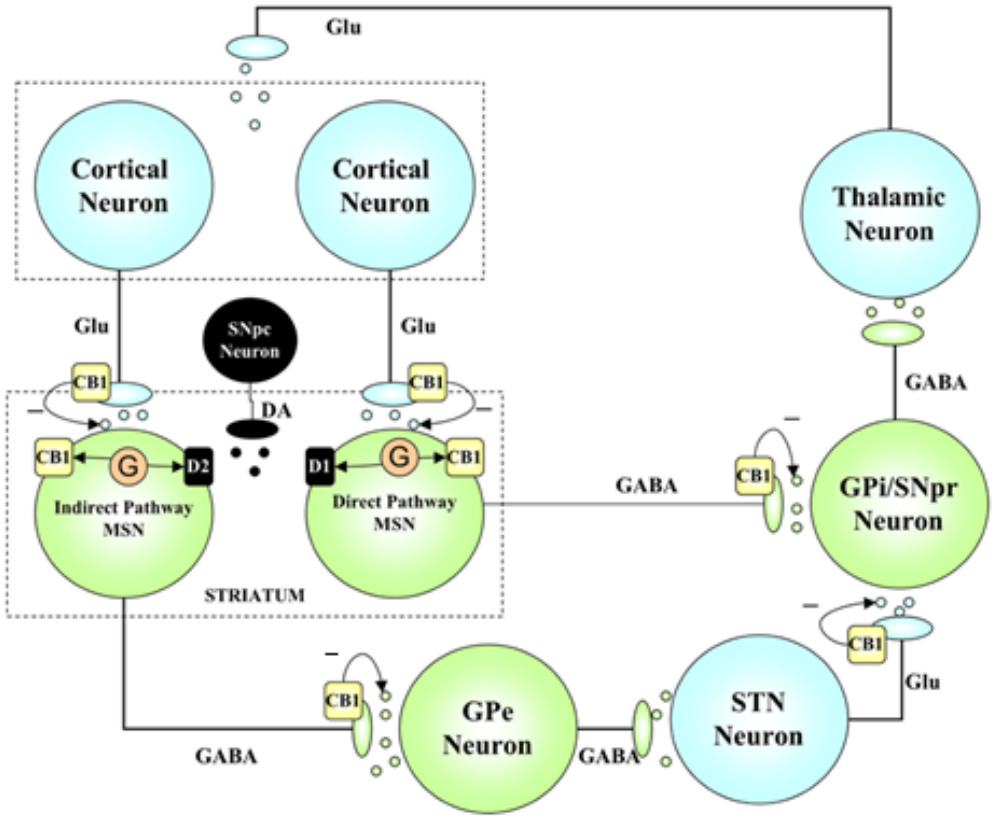

Figure 13. Expression and physiological function of CB1R within the basal ganglia circuitry. CB1R is expressed by striatal MSNs both in their soma and in their presynaptic axon terminals innervating the external and internal segments of the GP and the SNr, and are also present in corticostriatal excitatory terminals and in excitatory projections from the STN to the GPi/SNr and SNc .Activation of presynaptic CB1R located on corticostriatal terminals reduces glutamate release. Similarly, in the output basal ganglia nuclei (GPi and SNr), CB1R activation inhibits both glutamate release from STN afferents and GABA release from striatal afferents. In the striatum, a population of CB1R is co-expressed with D1R and D2R and shares with these two receptors a common pool of G proteins, suggesting a convergence of their signal transduction mechanisms. DA, dopamine; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulate. Adapted from Di Filippo et al. 2008.

Due to the direct modulation of MSNs synapses activity through eCBs, they represent one of the neurochemical substrates for the cross-talk between direct and indirect pathways (Fig. 13). The role of eCBs in the control of LTD is a major discussion issue in this context. Although this effect is observed in most MSNs, suggesting a lack of segregation in a specific pathway, the inhibition of glutamate release by retrograde eCB signalling is frequency-dependent and D2R-mediated (Kreitzer & Malenka 2007; Shen et al. 2008; Calabresi et al. 2014). However, other studies demonstrating that eCB-LTD can be induced in both MSNs subpopulations have challenged this view (Wang et al. 2006; Bagetta et al. 2011). Even though this question is not yet clear, it appears that there may be preferential LTD at synapses onto D2-MSNs with less robust induction protocols, but LTD can certainly be induced at synapses onto D1-MSNs with sufficiently strong synaptic activation. Overall, there is general agreement that CB1R receptors are expressed on afferents that innervate both D1-MSNs and D2-MSNs (Lovinger & Mathur 2010).

CB1R AND eCBs IN CNS DYSFUNCTION

eCB mediated neuroprotection

The eCB system represents an endogenous neuroprotective network in models of both **acute neuronal damage**, as stroke and traumatic brain injury (Panikashvili et al. 2001; Schurman & Lichtman 2017), and **neurodegenerative diseases**, such as multiple sclerosis, Parkinson´s disease, Huntington´s disease, Alzheimer´s disease and amyotrophic lateral sclerosis (Pryce et al. 2013; Fernández-Ruiz, Romero, et al. 2015). The fact that components of this system are found altered in many neurological diseases and that eCBs levels are enhanced upon brain damage, in addition to their broad-spectrum activity, gave rise to the idea of use them as pharmacological targets against brain degeneration (Mechoulam et al. 2014; Di Marzo et al. 2015). Due to the promising neuroprotective potential of eCBs, several studies have attempted to dissect the mechanistic bases of a plethora of eCB-mediated actions. Different strategies to approach this issue have been used, including **pharmacological treatments** and **genetic manipulation** of eCB system components. For example, by means of cannabinoid-drug treatments, it has been shown that THC protects hippocampal neurons from excitotoxicity (Gilbert et al. 2007), 2-AG is neuroprotective against brain injury (Panikashvili et al. 2001), WIN-55,212-2 and eCBs counteract neurodegeneration in models of Multiple sclerosis (Pryce et al. 2003; Loría et al. 2010), and AEA prevents neuronal death after excitotoxic damage (van der Stelt et al. 2001), among to many others.

In addition to those pharmacological studies, genetic manipulation of the eCB system has demonstrated that the lack of CB1R (CB1R knockout mice; **CB1R-KO**) renders mice more sensitive to excitotoxic damage compared to wild-type (**WT**) littermates (Parmentier-Batteur et al. 2002; Marsicano et al. 2003). On general grounds, CB1R-mediated neuroprotection usually involves synaptic modulation (Katona & Freund 2008), while **CB2R**-mediated neuroprotection is more likely related to resolving excessive neuroinflammatory events that come along with most neurodegenerative and acute-brain damage processes (Palazuelos et al. 2009; Valdeolivas & Satta 2012; Scheller & Kirchhoff 2016), although it also participate in other cell survival mechanism (Fernández-Ruiz et al. 2007). As mentioned before, CB2R is expressed in various circulating and resident immune cells, particularly when these cells are overactivated, and its engagement is typically associated with a reduction in it pro-inflammatory activity. This includes the inhibition of the release of inflammatory mediators, including **nitric oxide** (**NO**), **interleukin-2** (**IL-2**) and **TNF-α**, the inhibition of some cell-mediated immune processes, and the inhibition of proliferation and chemotaxis (Walter & Stella 2004).

A common experimental approach to modulate eCB levels involves the use **inhibitors** of their **degradation**, which provides a means of elevating eCB levels and therefore of activating cannabinoid receptors in a more prolonged fashion and with higher sensitivity to physiological, on-demand regulation, than acute administration of cannabinoid receptor agonists (Galve-Roperh et al. 2008; Di Marzo et al. 2015). This strategy can mimic the beneficial neuromodulatory effects of CB1R activation and the anti-inflammatory effects of CB2R activation in a time- and tissue-selective manner. For example, using **FAAH** inhibitors in mouse models it is possible to diminish the spasticity associated to multiple sclerosis (Pryce et al. 2013), and provide neuroprotection against excitotoxicity (Karanian et al. 2005).

The other principal eCB-degradative enzyme, **MGL**, has been also targeted, resulting in neuroprotection in models of Alzheimer´s disease (Chen et al. 2011) and Parkinson´s disease (Mounsey et al. 2015), among others. Interestingly, in both MGL KO mice and in animals treated with a MGL inhibitor, besides the expected increase in brain 2-AG levels, and therefore the promotion of cannabinoid receptor-mediated neuroprotection, it has been found that a profound decrease in the levels of free **AA**, as well as several eicosanoids, including **PGE2**, **PGD2**, **PGF2α** and **thromboxane B2**, occurs (Nomura et al. 2011). In addition, upon MGL inhibition or genetic deletion, the rise in brain eicosanoids and inflammatory cytokines

found upon LPS injection is prevented (Nomura et al. 2011; Grabner et al. 2016). In these studies, the reduction in activated cytokines was not reversed by CB1R antagonists (Grabner et al. 2016)., but was mimicked by **COX-1** blockade (Nomura et al. 2011), thus suggesting that they were due to changes in prostaglandin synthesis and COX-1 metabolites, but not to CB1R activation. On the other hand, **COX-2** can also produce proinflammatory mediators by using 2-AG as a substrate (Alhouayek & Muccioli 2014; Hermanson et al. 2014). As this enzyme is elevated in most neuroinflammatory processes, 2-AG accumulation might be harmful in some contexts or time points along damage progression (Van Der Stelt et al. 2006; Valdeolivas et al. 2013). 2-AG metabolism is not only a neuronal MGL-associated function, but most likely depends on the metabolic interplay between neurons and astrocytes, by which it provides a concerted oversight of 2-AG and, by doing so, allows protection of the nervous system from excessive CB1 receptor activation and promotes eCB crosstalk with other lipid transmitter systems (Viader et al. 2015). All these issues being considered, it is likely that MGL is an important enzyme involved in regulating synaptic 2-AG-based signalling as well as in controlling brain eicosanoid production (Figure 14).

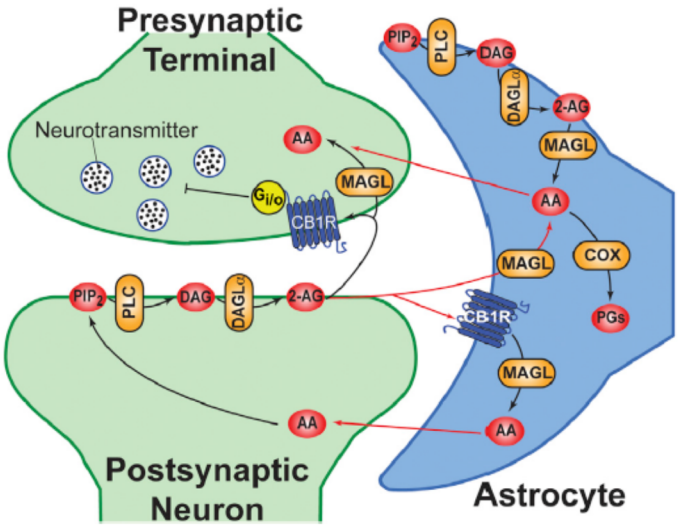

Figure 14. Distributed oversight of 2-AG metabolism and function in neurons and astrocytes. Proposed model of astrocytic-neuronal transcellular shuttling and metabolism of 2-AG and AA. PIP2, phosphatidylinositol 4,5-*bis*phosphate; COX, cyclooxygenase. Adapted from Viader et al. 2015.

CB1R-mediated neuroprotection

Endocannabinoid signalling serves as a major feedback mechanism to prevent excessive presynaptic activity and thus fine tunes the functionality and plasticity of many synapses, especially glutamatergic and GABAergic (Katona & Freund 2008; Kano et al. 2009). In concert with this neuromodulatory function, and as advanced above, studies in various animal models support that the CB1R plays an important role in the promotion of neuron survival in pathophysiological settings such as acute brain injury (Shohami et al. 2011) and neuroinflammatory conditions (Croxford et al. 2008).

Most of the neuroprotective activity of CB1R is believed to rely on the attenuation of **glutamatergic excitotoxicity** (Katona & Freund 2008) (Figure 15). eCBs can modulate glutamatergic signalling by different mechanisms, but the more extensively studied implies the activation of CB1R located on glutamatergic synaptic terminals, where it inhibits neurotransmitter release (Marsicano et al. 2003; Monory et al. 2006; Chiarlone et al. 2014) (Figure 11). Although GABAergic axon terminals contain many more CB1R molecules than their glutamatergic counterparts, they are not involved in seizure susceptibility (Monory et al. 2006) or protection against excitotoxicity (Marsicano et al. 2003; Chiarlone et al. 2014). Instead, those GABAergic CB1R pools play a pivotal role in THC-induced long-term memory deficits (Puighermanal et al. 2009), and protection against age-related cognitive decline (Albayram et al. 2011). Finally, since **astrocytes** participate in eCBs signalling, specifically in astrocyte-neuron communication through CB1R (Serrano et al. 2006; Navarrete & Araque 2008), and are central players in synaptic glutamate homeostasis (Rothstein et al. 1996), they could also conceivably influence neuronal damage upon glutamatergic excitotoxicity. In fact, in the hippocampus, the maintenance of epileptic discharges is reduced when the neuron-to-astrocyte communication via CB1R is pharmacologically blocked (Coiret et al. 2012).

CB1R also can exert **autocrine neuroprotective functions**, for example triggering intracellular signal transduction events that promote cell survival. In this context, the **PI3K/Akt** and the **ERK** pathways have been associated to eCB-mediated neuroprotection both *in vitro* (Gómez del Pulgar et al. 2000; Molina-Holgado et al. 2005; Blázquez et al. 2015; Batista et al. 2016) and *in vivo* paradigms (Ozaita et al. 2007; Blázquez et al. 2015).

A key downstream signalling target of CB1R is the serine/threonine kinase **mammalian target of rapamycin** (**mTOR**), a molecular integrator that enables both cellular nutrient sensing and energy homeostasis through the ERK/MAPK, Akt and AMPK pathways. The acute injection of THC induces a rapid and transient stimulation of mTOR complex 1 (mTORC1) activity in the hippocampus, striatum, cerebellum, frontal cortex and amygdala, whereas repeated administration of THC leads to more

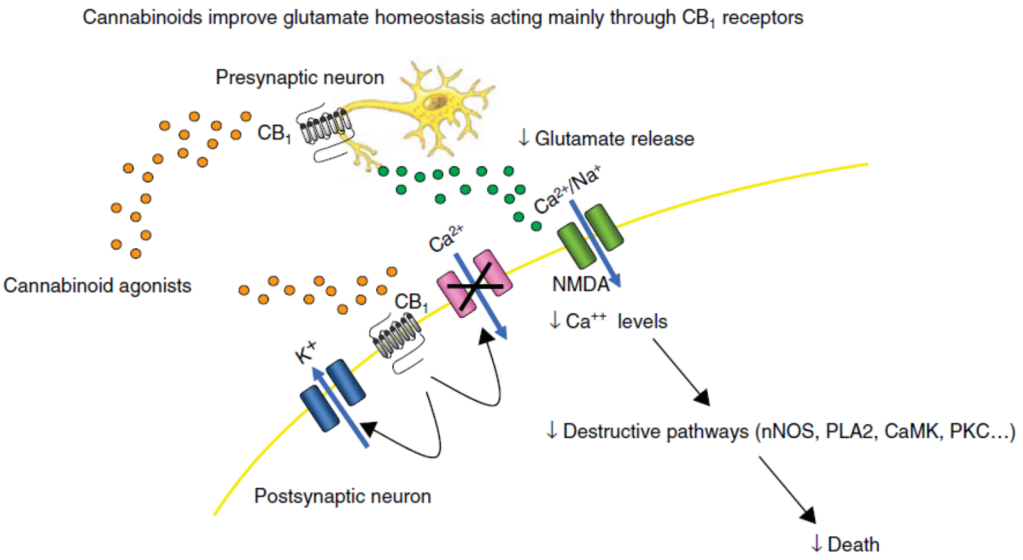

Figure 15. Schematic representation of mechanisms underlying the glutamatergic and calcium-blunting properties of cannabinoids that may serve to provide neuroprotection in different pathological conditions. Adapted from Fernández-Ruiz et al. 2010

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sustained activation of mTORC1, lasting for several days after the cessation of the treatment (Puighermanal et al. 2009; Puighermanal et al. 2013). In addition, mTORC1 is activated upon increased eCB tone (Busquets-Garcia et al. 2011), and is involved in neural progenitor cell proliferation (Díaz-Alonso et al. 2015) and oligodendrocyte differentiation (Gomez & Le 2011). The regulation of mTORC1 by CB1R is remarkably wide-ranging, as mTORC1 has also been implicated as a central regulator of autophagy, a cellular response that is believed to contribute to the ageing-associated loss of protein homeostasis. In fact, it has been shown that CB1R engagement protects MSNs from excitotoxic death via the PI3K/Akt/mTORC1 pathway, which, in turn, induces **brain-derived neurotrophic factor** (**BDNF**) expression through the selective activation of BDNF gene promoter IV, an effect that is mediated by multiple transcription factors (Blázquez et al. 2015).

CB1R and eCBs in motor diseases

The levels of some eCB system components, and in particular CB1R, are significantly altered in various BG disorders. Such alterations have been observed in samples obtained from patients (Glass et al. 1993; Glass et al. 2000; Lastres-Becker, Cebeira, et al. 2001) and animal models (Lastres-Becker, Cebeira, et al. 2001; Centonze et al. 2005; Bisogno et al. 2008; Fernández-Ruiz, Romero, et al. 2015). These findings, together with the neuroprotective and neuromodulatory role of eCBs in the BG, have prompted the search for cannabinoids-based therapies as a treatment of BG disorders.

In general, Parkinson's disease (**PD**) and Huntington's disease (**HD**) are the two basal ganglia disorders that have attracted most attention with regard to the potential clinical application of cannabinoids (Fernández-Ruiz et al. 2011). In this context, profound modifications in eCB signalling after dopamine depletion occur in experimental models of PD and patients. For example, in PD models, striatal levels of AEA are increased (Gubellini et al. 2002), which is associated with an increased spontaneous glutamatergic activity recorded from the large majority of MSNs, and this is reversed by L-DOPA treatment (Maccarrone et al. 2003). In addition, It has been demonstrated that eCB-LTD is selectively lost at indirect pathway MSN synapses, and that it can be rescued either in the presence of a D2R agonist or by the application of a FAAH inhibitor (Kreitzer & Malenka 2007). Also in HD, eCB levels suffer different alterations (Lastres-Becker, Fezza, et al. 2001; Bisogno et al. 2008; Centonze et al. 2005; Bari et al. 2013).

Of note, CB2R is overexpressed by glial and peripheral immune cells from HD models (Sagredo et al. 2009; Palazuelos et al. 2009; Bouchard et al. 2012) as well as in post-mortem tissues from HD patients (Palazuelos et al. 2009) and, therefore, cannabinoid compounds that activated both CB1R and CB2R seems to be effective in HD, mostly by counteracting excitotoxicity and inflammation (Sagredo et al. 2009; Chiarlone et al. 2014; Fernández-Ruiz, Moro, et al. 2015) (Figure 15). In addition, CB2R has been proposed has a neuronal loss marker in PD, since it is present in the substantia nigra (the principal neurodegenerative area in PD) at levels significantly lower in PD patients compared to controls (García et al. 2015).

In both disorders, early presymptomatic phases, characterized by neuronal malfunctioning, are associated with alterations of CB1R (Denovan-Wright & Robertson 2000; Glass et al. 2000; Blázquez et al. 2011) (Fig. 16). The symptomatic phases are characterized by opposite changes in both disorders.
In early HD, a profound loss of CB1R occurs, concomitant with MSN dysfunction, which is compatible with the hyperkinetic/choreic symptoms typical of these patients (Pazos et al. 2008). By contrast, a significant up-regulation of CB1R is found in early PD, which might constitute an adaptive response and is also compatible with the patients' akinetic/rigid clinical profile (Fernández-Ruiz et al. 2011) (Fig. 16).

Importantly, genetic deletion of CB1R aggravates the symptoms in HD mouse models (Mievis et al. 2011; Blázquez et al. 2011), while THC treatment (Blázquez et al. 2011) or rescue of CB1R expression improves at least some neuropathological aspects of the disease (Naydenov et al. 2014; Blázquez et al. 2015). In this context, recent studies have pointed out an unbalance in the glutamatergic and GABAergic CB1R pools in HD models. It is known that CB1R is lost in HD transgenic mice in a brain region-specific manner, as it occurs in the striatum but not in the cortex (Denovan-Wright & Robertson 2000; Lastres-Becker et al. 2002; McCaw et al. 2004). In fact, while CB1R located in MSNs is dramatically downregulated, thus turning these cells insensitive to receptor agonists, CB1R located on glutamatergic terminals remains unaltered (Chiodi et al. 2012; Chiarlone et al. 2014). Moreover, CB1R located specifically on glutamatergic corticostriatal projections, rather than on MSNs, has been found to constitute a central player in eCB-dependent neuroprotection from excitotoxicity- and mtHtt-induced damage (Chiarlone et al. 2014). Likewise, there is an increasing number of studies suggesting that CB1R pools located on different cell populations, and even intracellular compartments, may account for the observed distinct responses to eCB action (Busquets Garcia et al. 2016).

Therefore, dissecting the cell population-specific role of each CB1R pool seems crucial to understand the underlying eCB-mediated neuroprotective mechanisms, as well as to pave the way to the possible design of CB1R-targetted neuroprotective therapeutic strategies. This issue constitutes a central conceptual focus of the present PhD Thesis.

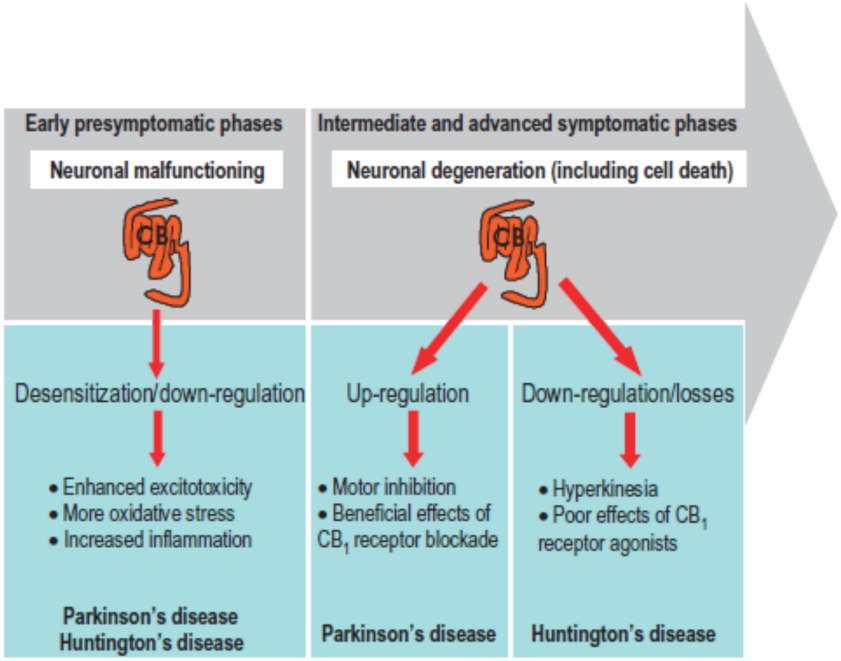

Figure 16 Changes of CB1R expression in the BG during the progression of Parkinson's and Huntington's diseases. Adapted from Fernández-Ruiz 2009

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AIMS

A key unanswered question in most neurodegenerative diseases is what precise factors dictate the selective damage of a particular neuronal population. The use of transgenic animals and/or recombinant adeno-associated virus (rAAV)-mediated gene delivery allows to express the DREADD in a particular cell population of a given brain region, offering a powerful strategy to solve cell-specific mechanism in health and disease. Specifically in HD, a neurodegenerative disease where the primarily affected structures are the cortex and striatum, the mechanisms by which striatal principal neurons (MSNs) are so highly vulnerable are incompletely understood. Between the plethora of neurotransmitter systems, the endocannabinoid system plays a pivotal role in striatal function and present a broad spectrum of neuroprotective actions. However, despite the widely reported neuroprotective role of the CB1 receptor, the assessment of its physiological relevance and therapeutic potential in neurological diseases is hampered, at least in part, by the lack of knowledge of the cell-population specificity of CB1R action.

In this context, the **GLOBAL AIM** of this Doctoral Thesis embraces the study of direct versus indirect pathway mechanisms involved in corticostriatal dysfunction and neurodegeneration, as well as the selective neurobiological site of CB1R neuroprotective action within the corticostriatal circutry.

This main objective can be divided into 2 **SPECIFIC AIMS**:

Objective 1. Examine the molecular mechanisms and physiopathological relevance of Gq-protein signalling in direct pathway and indirect-pathway MSNs.

Objective 2. Study the role of different CB1R pools, namely those situated on GABAergic neurons (MSNs), glutamatergic neurons (corticostriatal projection neurons) or astrocytes, in the differential vulnerability of D1R- and D2R-MSNs.

MATERIALS AND METHODS

MATERIALS AND METHODS-OBJECTIVE 1

Animals

We used mutant mice and their corresponding wild-type littermates in which Cre recombinase expression was driven by the D1R promoter (Monory et al. 2007); colony founders provided by Günther Schütz, German Cancer Research Center), the D2R promoter (colony founders provided by University of California Davis Knockout Mouse Project Repository, Davis, CA), or both the D1R and D2R promoters (generated by crossing the aforementioned D1R-Cre and D2R-Cre mouse lines). All lines were in the C57BL/6N background. Wild-type C57BL/6N mice were purchased from Harlan Laboratories. Animal housing, handling, and assignment to the different experimental groups were conducted as described previously (Blázquez et al. 2011). All experimental procedures used were performed in accordance with the guidelines and with the approval of the Animal Welfare Committee of Madrid Complutense University according to the European Commission directives.

Viral vectors

Gq-coupled human M3 muscarinic DREADD (hM3Dq) fused to mCherry (provided by Brian L. Roth, University of North Carolina, Chapel Hill, NC; (Alexander et al. 2010) or mCherry alone was subcloned in a recombinant adeno-associated virus (rAAV) expression vector with a minimal CAG promoter (for generalized expression) or in a CAG-DIO vector (for Cre-dependent expression) using standard molecular biology techniques. For cell-specific ablation, a mCherry-FLEX-DTA cassette (Addgene plasmid #58536, provided by Naoshige Uchida, Harvard University) was cloned in a CAG-DIO-rAAV vector. All vectors used were of an AAV1/AAV2 mixed serotype and were generated by calcium phosphate transfection of HEK-293T cells and subsequent purification as described previously (Monory et al. 2006).

DREADD-induced neuronal manipulation in vivo

Eight-week-old male C57BL/6N mice were injected stereotaxically with CAG-hM3Dq-mCherry-rAAV or control CAG-rAAV (in 1.5 µl of PBS) aimed at targeting the dorsal striatum. Each animal received 1 bilateral injection at the following coordinates (to bregma): anteroposterior +0.5, lateral ±2.0, and dorsoventral ±3.0. Four weeks after surgery, mice were assigned to different experimental groups before starting the pharmacological treatments. Rotarod performance was analyzed along the last 3 d of treatment. Mice were subsequently killed by intracardial perfusion and their brains were excised for immunofluorescence analyses.

Eight-week-old wild-type, D1R-Cre, D2R-Cre, and/or D1R/D2R-Cre mice were injected either unilaterally into the right brain hemisphere (for assessing rotational behavior) or bilaterally (for assessing motor activity, motor coordination, and sleep–wake pattern) with the Cre-dependent CAG-DIO-hM3Dq-mCherry-rAAV at the aforementioned coordinates. Animals were left untreated for 4 weeks after surgery before the pharmacological treatments and behavioral tests (see below).

Drug administration in vivo

CNO (Santa Cruz Biotechnology) was prepared fresh in saline (0.9% NaCl) just before the experiments and injected intraperitoneally at 1 or 10 mg/kg. SP600125 (2H-dibenzo [cd,g]indazol-6-one) was dissolved in 45% (w/v) β-cyclodextrin (Sigma-Aldrich) and injected intraperitoneally at 15 mg/kg.

Behavioral and electroencephalographic assays Spontaneous locomotor activity

D1R-Cre, D2R-Cre, and D1R/D2R-Cre mice and their wild-type littermates were injected bilaterally with CAGDIO-hM3Dq-mCherry-rAAV as described above. After vector administration, passive retro-reflective markers (B&L Engineering; diameter 7.9 mm, weight_0.5 g) were attached with acrylic dental cement to the skull of each mouse, which was single-housed in its cage. Acquisition (5 Hz frequency) was performed using 3 OptiTrack Flex3 cameras (Natural Point), allowing the continuous recording of the position of each animal during dark and light phases. Acquisition and automated tracking software were from MouvTech. Throughout the study, animals had unrestricted access to water and food and were subjected to a 12 h light/12 h dark cycle. Offline analysis was performed using homemade software developed with Matlab (The MathWorks). Mice were habituated to the home cage for 7 d. They were then injected with vehicle (saline), followed 24 h later by acute CNO (1 mg/kg) and then by chronic CNO (10 mg/kg/d for 14 d). Total locomotor activity in 12 h light/12 h dark cycles was recorded.

Exploration, motor coordination, and spatial recognition

D1R-Cre, D2R-Cre, and D1R/D2R-Cre mice and their wild-type littermates were injected bilaterally with CAG-DIO-hM3Dq-mCherry-rAAV as described above. They underwent a treatment schedule of 1 d of acute

CNO (1 mg/kg) followed by chronic CNO (10 mg/kg/d for 14 d) (or saline vehicle). Exploration analyses were conducted in an automated actimeter (ActiTrack; Panlab; (Blázquez et al. 2011) the first day of acute treatment (1 h after injection), as well as after the last day of treatment. Motor coordination (Rotarod test) and spatial recognition (Y-maze test) were evaluated along the last 3 d of treatment before CNO administration to avoid acute drug effects (Blázquez et al. 2011; Pietropaolo et al. 2014).

Sleep–wake pattern

D1R-Cre, D2R-Cre, and D1R/D2R-Cre mice and their wild-type littermates were injected bilaterally with CAG-DIOhM3Dq-mCherry-rAAV as described above and implanted a multisite electrode array for electroencephalographic recordings as described previously (Lebreton et al. 2015). Mice underwent two sessions of acute activation recording, one with vehicle (saline) and another, after 24 h, with CNO (1 mg/kg) plus one session of chronic activation recording after the last day of chronic CNO treatment (10 mg/kg/d for 14 d or saline vehicle). Electrophysiological data were analyzed as described previously (Lebreton et al. 2015).

Rotational behavior

D1R-Cre and D2R-Cre mice were injected unilaterally with CAG-DIO-hM3Dq-mCherry-rAAV as described above. Mice were then acutely injected with vehicle (saline) or, after 24 h, with CNO (1 mg/kg); in both cases, animals were tested 1 h later in an open field. Subsequently, animals were injected for 15 d with CNO (10 mg/kg/d) together with SP600125 (15 mg/kg/d) or their respective vehicles. One day after the last treatment, all mice were injected with vehicle and, after 24 h, with CNO (1 mg/kg); in both cases, animals were tested 1 h later in an open field. Ipsilateral movements (complete turning to the right) and contralateral movements (complete turning to the left) were assessed by monitoring manually the total time spent in rotation for 5 min. No rotations were observed in wild-type mice expressing CAG-DIOhM3Dq-rAAV and injected with vehicle or CNO.

Immunomicroscopy

Coronal free-floating sections (50 µm-thick) were obtained from paraformaldehyde-perfused mouse brains. Samples were incubated with antibodies against dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32; 1:1000; BD Biosciences, #611520), NeuN (1:500; Millipore, #MAB377), D1R (1:500; Frontier Science, #af500), D2R (1:500; Frontier Science, #af750), choline acetyltransferase (ChaT; 1:1000; Merck, #AB144P) or cFos (1:2000; Santa Cruz Biotechnology, #SC52), followed by staining with the corresponding Alexa Fluor 488, 594, or 647 antibodies (1:1000; Life Technologies) or with HRP-coupled secondary antibodies plus DAB chromogenic visualization (Vector Laboratories) (Blázquez et al. 2011). Nuclei were visualized with DAPI. Analysis of marker protein immunoreactivity in the dorsal striatum was conducted in a 1-in-10 series per animal (from bregma 1.5 to ± 0.5 coronal coordinates). For DARPP-32, D1R, and D2R, data were calculated as immunoreactive area per total cell nuclei and are expressed as a percentage of the control. D1R and D2R immunofluorescence was counted simultaneously, so these are shown in the same samples. For NeuN and ChaT, data were calculated as number of positive cells per total cell nuclei and expressed as percentage of the control. Confocal fluorescence images were acquired using TCS-SP2 software and a SP2 AOBS microscope (Leica). Pixel quantification and colocalization were analyzed with ImageJ software.

Western blotting

Western blot analysis was conducted with antibodies raised against phosphorylated ERK (1:1000; Cell Signaling Technology, #9101), total ERK (1:1000; Cell Signaling Technology, #9102), phosphorylated JNK (1:1000; Cell Signaling Technology, #9255), total JNK (1:1000; Cell Signaling Technology, #9252), phosphorylated cJun (1:1000; Cell Signaling Technology, #2361), total cJun (1:1000; Cell Signaling Technology,#9156), phosphorylated PYK2 (1:1000; Cell Signaling Technology, #3291), total PYK2 (1:1000; Cell Signaling Technology, #3480), and β-tubulin III (1:4000; Sigma-Aldrich, #T8660), following standard procedures (Blázquez et al. 2011). Densitometric analysis was performed with Quantity One software (Bio-Rad).

Cell culture

Conditionally immortalized mouse striatal neuroblasts infected with a defective retrovirus transducing the temperature-sensitive A58/U19 large T antigen (Trettel et al. 2000), designated as STHdh cells; provided by Silvia Ginés, University of Barcelona, Spain) were grown at 33°C in DMEM supplemented with 10% fetal bovine serum, 1mM sodium pyruvate, 2 mM L-glutamine, and 400 µg/ml geneticin (Blázquez et al. 2011). Cells were devoid of mycoplasma contamination. Primary striatal neurons were obtained from 2-d-old C57BL/6N mice using a papain dissociation system (Worthington). Striata were dissected and cells were seeded on plates precoated with 0.1 mg/ml poly-D-lysine at 200,000 cells/cm² in

Neurobasal medium supplemented with B27 and Glutamax (Blázquez et al. 2011).

Cell nucleofection and infection

STHdh cells were nucleofected with a construct expressing hM3DqmCherry (or mCherry alone as control) under the CAG promoter (see above) using an Amaxa mouse neuron nucleofector kit (Lonza). Two days after nucleofection, cells were treated in 0.5% FBS medium with CNO (or H_2O as vehicle) in the presence of different signaling pathway inhibitors. Cells were treated for up to 8 h for cell viability or Western blot assays (Blázquez et al. 2011). In a second set of experiments, the CAG-hM3Dq-mCherry construct was conucleofected with plasmids expressing shRNA directed to Jnk1, Jnk2, Jnk3, or Pyk2 or a scrambled control (Origene). The extent of silencing induced by the different kinase-directed shRNA, as determined by RT-PCR, ranged between 50% and 80% relative to the scrambled control. In a third set of experiments, STHdh cells were nucleofected with a CAG-DTA construct. Primary neurons were infected at day 2 in vitro with a rAAV expressing hM3Dq (or GFP as control) and kept until day 13 in vitro for cell viability experiments.

Statistics

Data are presented as mean ± SEM. Statistical comparisons were made by one-way or two-way ANOVA with post hoc Bonferroni, Tukey, or Neuman–Keuls test or by unpaired Student's t test. A p value of less than of 0.05 was considered significant. Graphs and statistics were generated by GraphPad Prism 6.01.

MATERIALS AND METHODS-OBJECTIVE 2

Animals

We used conditional mutant mice, generated by the Cre-loxP technology, in which the CB1R gene is primarily absent from cortical glutamatergic neurons of the dorsal telencephalon ($CB1R^{floxed/floxed;Nex\text{-}Cre/+}$ mice; herein referred to as Glu-$CB1R^{-/-}$ mice) (Monory et al. 2006) or from astroglial cells ($CB1R^{floxed/floxed;GFAP\text{-}CreERT2/+}$ mice; herein referred to as GFAP-$CB1R^{-/-}$ mice; treated with tamoxifen to induce Cre expression as described) (Han et al. 2012). We also used mice bearing a genetic deletion of the MGL gene in all body cells (either in heterozygous or homozygous form; $MGL^{+/-}$ and $MGL^{-/-}$ mice, respectively) or selectively in astroglial cells ($MGL^{floxed/floxed;GFAP\text{-}Cre/+}$ mice; herein referred to as GFAP-$MGL^{-/-}$ mice) (Grabner et al. 2016). BAC transgenic mice expressing the tdTomato and EGFP reporter genes under the control of the D1R and D2R promoter, respectively (*Drd1a*-tdTomato/*Drd2*-EGFP mice), were also used (colony founders kindly provided by Dr. Rosario Moratalla, Cajal Institute, Madrid, Spain). Hemizygous mice transgenic for exon 1 of the human huntingtin gene with a largely expanded CAG tract (~250 CAG repeats; R6/2L mice) were generated from R6/2 mice (The Jackson Laboratory) and subsequently crossed with $CB1R^{floxed/floxed}$ mice to obtain the double-mutant R6/2L:$CB1R^{floxed/floxed}$ line as described (Chiarlone et al. 2014). Wild-type C57BL/6N mice were purchased from Harlan Laboratories. In all the experiments, mice were compared with their corresponding littermates. Animal housing, handling and assignment to the different experimental groups were conducted as described (Blázquez et al. 2011). Mice were sacrificed by intracardial perfusion and their brains were excised for tissue analyses. All the experimental procedures used were performed in accordance with the guidelines and with the approval of the Animal Welfare Committee of Universidad Complutense de Madrid and Comunidad de Madrid, and in accordance with the directives of the European Commission.

Viral vectors

Constructs expressing CFP-tagged human huntingtin exon 1 harboring a pathogenic polyQ tract of 94 CAG repeats or a normal, non-pathogenic polyQ tract of 16 CAG repeats (Maynard et al. 2009) (kindly provided by Dr. José J. Lucas, Severo Ochoa Molecular Biology Center, Madrid, Spain), HA-tagged Cre recombinase (Monory et al. 2006), EGFP (Chiarlone et al. 2014) or Gq-coupled human M3 muscarinic DREADD (hM3Dq) fused to mCherry (Alexander et al. 2010) (kindly provided by Dr. Brian L. Roth, University of North Carolina, Chapel Hill, NC), were subcloned in rAAV expression vectors with a minimal CaMKIIα or GFAP promoter (kindly provided by Dr. Karl Deisseroth, Stanford University, Stanford, CA) by using standard molecular cloning techniques. All vectors used were of an AAV1/AAV2 mixed serotype, and were generated by polyethylenimine (PEI) transfection of HEK293T cells and subsequent purification (Monory et al. 2006). Vectors were injected stereotactically either into the dorsal striatum (vectors diluted in 3 µl PBS) or into the motor cortex projecting onto the dorsal striatum (vectors diluted in 1.5 µl PBS). In the case of the striatum, each animal received one bilateral injection at coordinates (to bregma): antero-posterior +0.5, lateral ±2.0, dorso-ventral -3.5. In the case of the cortex, each animal received 2 bilateral injections at coordinates (to bregma): antero-posterior +1.5, lateral ±1.2, dorso-ventral -1.7; and antero-posterior -0.5, lateral ±1.2, dorso-ventral -1.2. We have described previously the placement of the rAAV vectors within the cortex and the striatum under those conditions (Chiarlone et al. 2014; Blázquez et al. 2015; Bellocchio

et al. 2016). In the DREADD experiments (Chiarlone et al. 2014), animals were left untreated for 6 weeks after rAAV vector injection prior to starting the pharmacological treatments for 4 weeks, after which RotaRod test was conducted and animals sacrificed. In the R6/2L:CB1$^{floxed/floxed}$ mouse experiments (Chiarlone et al. 2014), 4 week-old animals were injected with the rAAV vectors and, at week 20 of age, RotaRod test was conducted and animals were subsequently sacrificed.

Drugs

CNO (Santa Cruz Biotechnology) was prepared fresh in saline (0.9% NaCl) just before the experiments and injected i.p. at 1 mg/kg/d. Stock solution of MK-801 (Sigma) was prepared in DMSO and, just before the experiments, diluted in saline (final DMSO concentration: 2%) for i.p. injections (0.03 mg/kg/d). SR141716 (rimonabant; kindly provided by Sanofi-Aventis, Montpellier, France), THC (The Health Concept), and JZL-184 (Cayman Chemical) were stored in DMSO. Just before the experiments, solutions of vehicle [1% (v/v) DMSO in Tween-80/saline (1:18, v/v)], SR141716 (1 mg/kg/d), THC (1 mg/kg/d) or JZL-184 (8 mg/kg/d) were prepared for i.p. injections. SKF-81297 (Tocris) was prepared fresh in saline just before the experiments and injected i.p. at 1 mg/kg.

Confocal microscopy

Coronal free-floating sections (50 µm-thick) were obtained from paraformaldehyde-perfused mouse brains. Samples were incubated with antibodies against NeuN (1:500; Chemicon #MAB377), DARPP-32 (1:1000; BD #611520), D1R (1:500; Frontier Science #af500), D2R (1:500; Frontier Science #af750), PSD-95 (1:500; Abcam #ab18258, S100β (1:500; Abcam #ab868), followed by staining with the corresponding Alexa Fluor 488, 594 or 647 antibodies (1:1000; Life Technologies). Nuclei were visualized with DAPI. Analysis of marker-protein immunoreactivity in the dorsal striatum was conducted as described (Bellocchio et al. 2016) in a 1-in-10 series per animal (from bregma +1.5 to -0.5 coronal coordinates). For DARPP-32, D1R, D2R and PSD-95, data were calculated as immunoreactive area per total cell nuclei, and expressed as percentage of the control. For NeuN, S100β, as well as for tdTomato and EGFP fluorescence in Drd1a-tdTomato/Drd2-EGFP mice, data were calculated as number of positive cells per total cell nuclei, and expressed as percentage of the control. Confocal fluorescence images were acquired using TCS-SP2 software and a SP2 AOBS microscope (Leica). Pixel quantification and co-localization were analyzed with ImageJ software (NIH, Bethesda, MD).

Behavior

Motor coordination (RotaRod performance) was evaluated along 3 consecutive days as described (Bellocchio et al. 2016). Exploration analyses were conducted in an automated actimeter (ActiTrack; Panlab) 30 min after acute treatment with vehicle or SKF-81297 (Bellocchio et al. 2016).

Microdialysis

Glutamate and GABA were measured from 8 week-old Glu-CB1R$^{-/-}$ mice or their CB1R$^{floxed/floxed}$ littermates. Fractions of dialysate were on-line analyzed for Glutamate content every 15 min using an HPLC system with electrochemical detection. The HPLC system consisted of a Waters 510 series pump in conjunction with an electrochemical detector (Mod. Intro, Antec, Leyden, The Netherlands). Dialysates were injected onto a 5 mm RP-18 column (LiChroCART 125-4, Merck, Darmstadt, Germany) via a VALCO valve fitted with a 65 µL sample loop. Detection limit was defined by a signal to noise ratio of 2:1, being approximately 6 fmol/sample.

Synaptosomes

Striatal synaptosomes were isolated from 8 week-old C57BL/6N mice as described (Martín et al. 2010). They were subsequently incubated with no additions, pertussis toxin (PTx) (1.5 mg/ml, 2 h), CCG-1423 (25 µM, 45 min) or YM-25489 (10 µM, 45 min). HU-210 (5 µM) was added to the synaptosomal preparations 1 min before the glutamate release measures. Glutamate concentration in the extracellular medium was assayed.

2-AG and AEA quantification

The levels of 2-AG and AEA were measured as described previously (Gomez et al. 2010) by HPLC/MS. Striata were isolated from 8 week-old MGL$^{+/+}$, MGL$^{-/-}$, GFAP-MGL$^{floxed/floxed}$ and GFAP-MGL$^{-/-}$ mice. Absolute 2-AG and AEA levels were estimated by comparison with their respective d8-2-AG and d8-AEA standards.

Statistics

Data are presented as mean ± SEM. Statistical comparisons were made by one-way or two-way ANOVA with post hoc Bonferroni, Tukey, or Sidak test or by unpaired Student's t test. A p value of less than of 0.05 was considered significant. Graphs and statistics were generated by GraphPad Prism 6.01.

RESULTS

CHAPTER 1

The dorsal striatum plays a key role in the control of motor behavior. The functionality of striatal neurons is tightly controlled by various metabotropic receptors. Whereas the Gs/Gi-protein-dependent tuning of striatal neurons is fairly well known, the precise impact and underlying mechanism of Gq-protein-dependent signals remain poorly understood. In this chapter, by using different experimental approaches, especially the DREADD technology, we show that sustained activation of Gq-protein signaling impairs the functionality of striatal neurons and unveil the precise molecular mechanism underlying this process, which involves a PLC/Ca^{2+}/PYK2/JNK pathway. Moreover, engagement of this intracellular signaling route is functionally active in the mouse dorsal striatum *in vivo*, as proven by the disruption of neuronal integrity and behavioral tasks. Acute Gq-protein activation in direct-pathway or indirect-pathway neurons produces an enhancement or a decrease, respectively, of activity-dependent parameters. In contrast, sustained Gq-protein activation impairs the functionality of direct-pathway and indirect-pathway neurons and disrupts the behavioral performance and electroencephalography-related activity tasks controlled by either anatomical framework. Collectively, these findings define the molecular mechanism and functional relevance of Gq-protein-driven signals in striatal circuits under normal and overactivated states.

CAPÍTULO 1

El estriado dorsal desempeña un papel clave en el control motor. La función de las neuronas estriatales está finamente controlada por varios receptores metabotrópicos. Mientras que la modulación de las neuronas del estriado dependiente de proteínas Gs/Gi es bastante conocido, el impacto exacto y el mecanismo subyacente de señales dependiente de la proteína Gq siguen sin comprenderse por completo. En este capítulo, utilizando diferentes aproximaciones experimentales, especialmente la tecnología DREADD, se muestra que la activación sostenida de la señalización a través de proteínas Gq daña la funcionalidad de las neuronas del estriado, y se revela el mecanismo molecular exacto que subyace a este proceso e involucra la vía de señalización de PLC/Ca^{2+}/PYK 2/JNK. Es más, el compromiso de esta ruta de señalización intracelular es también ocurre en el estriado dorsal de ratón *in vivo*, como demuestra la alteración de la integridad neuronal y las tareas conductuales. La activación aguda de las proteínas Gq en las neuronas de la vía directa o indirecta produce un aumento o una disminución, respectivamente, de parámetros dependientes de actividad. Por el contrario, la activación sostenida de proteínas Gq deteriora la funcionalidad de las neuronas de la vía directa e indirecta y altera tanto el comportamiento como el registro electroencefalográfico (EEG) derivado de la actividad motora controlados por estas dos vías anatómicas. Colectivamente, estos resultados definen el mecanismo molecular y la relevancia funcional de la señalización mediada por proteínas Gq en los circuitos estriatales durante estados normales y de sobreactivación.

RESULTS-OBJECTIVE 1

Sustained Gq-protein signaling disrupts the balanced control of behavior exerted by D1R-MSNs and D2R-MSNs in vivo

To study the impact of Gq-driven signaling on striatal circuits, we set up an experimental model to manipulate direct-pathway or indirect-pathway MSNs selectively and reliably in vivo. For this purpose, we first injected a FLEX (CAG-DIO) rAAV encoding mCherry into the dorsal striatum of D1R-Cre and D2R-Cre mice, which allowed delineating the connectivity to output nuclei (Fig. 1A). Counting of mCherry-positive cells in D1R-Cre and D2R-Cre mice showed that recombination was slightly higher in the former mouse line (63 ± 5% and 40 ± 4% of mCherry-positive cells in D1R-Cre and D2R-Cre mice, respectively; n = 7 animals per group). We also analyzed mCherry expression in ChaT-positive interneurons and found that our CAG-DIO-rAAV-driven infection procedure generated no detectable recombination in D1R-Cre mice (0% of ChaT-positive cells infected; n = 7 mice) and only a negligible recombination in D2R-Cre mice (<3% of ChaT-positive cells infected; n = 7 mice). Next, a CAG-DIO-rAAV encoding hM3Dq fused to mCherry was injected in the same experimental conditions. The Cre-driven expression of the transgene was achieved selectively in D1R-MSNs and D2R-MSNs, as evidenced by D1R/mCherry and D2R/mCherry colabeling analyses (Fig. 1B). Moreover, the ability of the transgene to trigger neuronal activation was proven by the enhanced cFos immunoreactivity observed in the striata of D1R-Cre and D2R-Cre mice (but not wild-type mice) that had been acutely treated with CNO [one single intraperitoneal injection at 1 mg/kg (Alexander et al. 2010) (Fig. 1C)].

In a first experimental paradigm aimed at assessing dorsal striatum functionality, we observed that acute activation of Gq-protein signaling in D1R-MSNs enhanced exploratory activity in an open field, whereas acute activation in D2R-MSNs produced the opposite effect (Fig. 2A). Strikingly, upon chronic CNO treatment [one daily intraperitoneal injection of CNO at 10 mg/kg for 14 d; (Alexander et al. 2010; Chiarlone et al. 2014)] the acute Gq-evoked hyperlocomotor reactivity on D1R-MSNs was abolished and the acute Gq-evoked hypolocomotor reactivity on D2R-MSNs did not only disappeared, but even turned to an opposite hyperlocomotor reactivity (Fig. 2B).

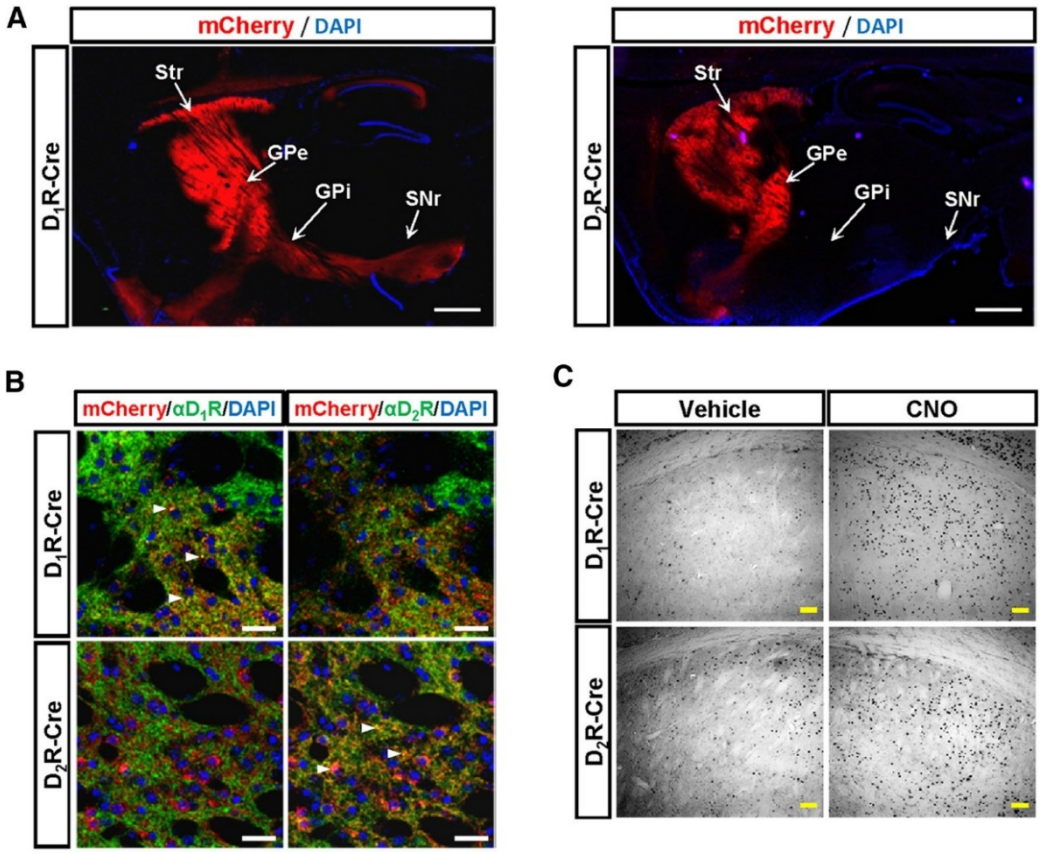

Figure 1. Expression and activity of hM3Dq-mCherry in D1R-MSNs and D2R-MSNs in vivo. D1R-Cre and D2R-Cre mice were injected stereotaxically into the dorsal striatum with CAG-DIO-mCherry- rAAV (A) or CAG-DIO-hM3Dq-mCherry-rAAV (B, C), and left untreated for 4 weeks. A, Representative images delineating the connectivity from the striatum to output nuclei in D1R-Cre and D2R-Cre mice. Str, Striatum; GPe, external globus pallidus; GPi, internal globus pallidus; SNr, substantia nigra pars reticulata. Scale bar, 1 mm. B, Fluorescence colabeling of D1R/mCherry and D2R/mCherry shows the specificity of Cre-driven recombination. Examples of cells that are double-positive for D1R/mCherry or D2R/mCherry are indicated by arrows. Scale bar, 30 µm. C, Animals subsequently received one single intraperitoneal injection of vehicle or CNO (1 mg/kg) and, after 2 h, were killed for cFos immunohistochemistry. Scale bar, 75 µm.

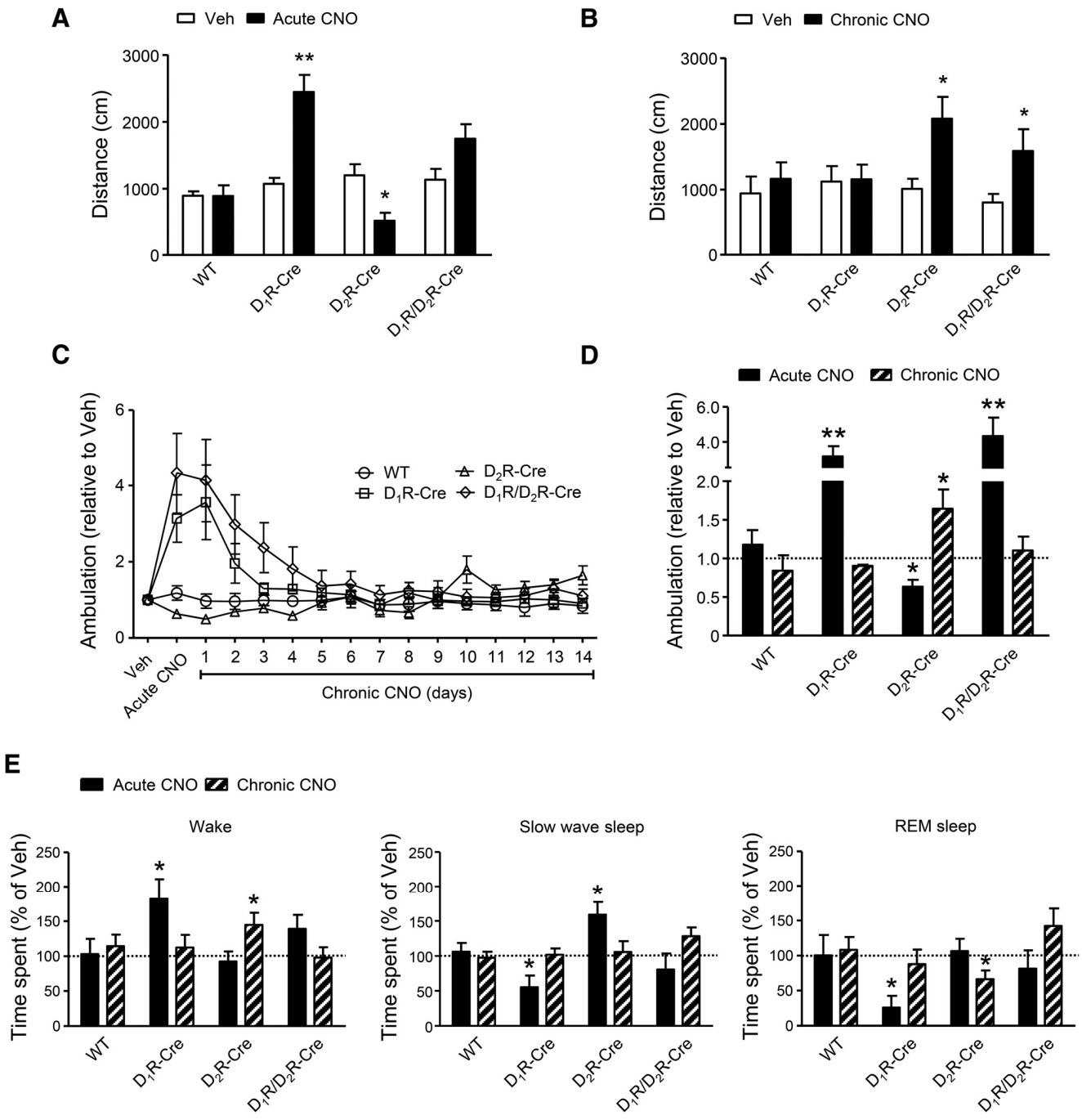

Figure 2. Sustained Gq-protein signaling disrupts the balanced control of behavior exerted by D1R-MSNs and D2R-MSNs in vivo. Wild-type, D1R-Cre, D2R-Cre, and D1R/D2R-Cre mice were injected stereotaxically into the dorsal striatum with CAG-DIO-hM3Dq-mCherry-rAAV and left untreated for 4 weeks. A, Animals subsequently received one single intraperitoneal injection of vehicle or CNO (1 mg/kg) and, 1 h later, their ambulation was tested in an open field. B, Animals subsequently received one daily intraperitoneal injection of vehicle or CNO (10 mg/kg) for 14 consecutive days and their ambulation was tested in an open field. C, D, Animals used for continuous ambulatory activity recordings were implanted during surgery light-reflecting devices. They subsequently received one single intraperitoneal injection of vehicle, followed after 24 h by CNO (1 mg/kg; acute CNO) and, after 24 h, one daily intraperitoneal injection of vehicle or CNO (10 mg/kg) for 14 consecutive days (chronic CNO). Total daily ambulations for the 12 h light period (7:00–19:00), expressed as percentage of vehicle treatment, are shown for 16 consecutive days (C), as well as for the acute day and the last chronic-treatment day (D). E, Animals used for electroencephalographic recordings were implanted during surgery with electrode-miniature array devices. They subsequently underwent 2 sessions of acute-activation electroencephalographic recording, one with vehicle (saline) and another, after 24 h, with CNO (1 mg/kg) plus one session of chronic-activation electroencephalographic recording after the last day of chronic CNO treatment (10 mg/kg/d for 14 consecutive days; or saline vehicle). In each case, animals were subjected to recording for 3 h and sleep–wake changes induced by CNO treatment were expressed as percentage of the respective vehicle treatment. *$p < 0.05$, **$p < 0.01$ from the corresponding vehicle group (A, B) or the wild-type group (D, E).

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In a second experimental paradigm, the effect of acute and chronic Gq activation in the direct or indirect pathway was monitored using passive retro-reflective markers attached to the head of each mouse expressing hM3Dq-mCherry in D1R-MSNs or D2R-MSNs. Singly housed mice were thus continuously tracked in their home cage during 16 consecutive days under vehicle (one single intraperitoneal saline injection), acute CNO (one single intraperitoneal injection at 1 mg/kg the day after) and subsequent chronic CNO (one daily intraperitoneal injection at 10 mg/kg for 14 d). Consistent with the aforementioned open-field data, acute Gq activation in D1R-MSNs increased continuous ambulatory activity, whereas acute Gq activation in D2R-MSNs led to the opposite outcome (Fig. 2C,D). Sustained Gq activation in D1R-MSNs abolished the acute hyperactivity, whereas sustained Gq activation in D2R-MSNs turned the acute hypoactivity into hyperactivity (Fig. 2C,D). These behavioral changes were visible only during light cycle (data not shown for dark cycle). The different response to chronic CNO treatment shown by D1R/D2R- Cre mice in Figure 2, C and D, versus Figure 2B most likely reflects the different type of test used, namely locomotor activity in the home cage versus locomotor reactivity in a novel environment. The latter can be subjected to other behavioral components such as anxiety and risk assessment, which, however, fall beyond the scope of the present study.

A third experimental paradigm was used to evaluate whether the aforementioned behavioral changes in activity were accompanied by actual electroencephalographic changes, specifically in the sleep–wake pattern. Therefore, we found that acute Gq activation in D1R-MSNs, in concert with hyperactivity, produced an increased (REM) sleep (Fig. 2E, right). Acute Gq activation in D2R-MSNs, in concert with hypoactivity, decreased time spent in both slow-wave amount of wake (Fig. 2E, left), as well as a (Fig. 2E, middle) and rapid eye movement induced an increased amount of sleep, specifically in the slow-wave phase (Fig. 2E, middle). Conversely, sustained Gq activation in D1R-MSNs abolished, not only the acute hyperactivity, but also the acute wakefulness state (Fig. 2E, left), whereas sustained Gq activation in D2R-MSNs not only induced hyperactivity, but also enhanced the time spent in wake (Fig. 2E, left) and reduced the time spent in REM sleep (Fig. 2E, right). Spectral analysis of electroencephalograms did not reveal changes in major brain rhythms in the delta, theta, or gamma frequencies during different vigilance states (data not shown).

Collectively, these findings show that direct-pathway and indirect-pathway MSNs can be "turned on" by acute Gq-protein signaling or "turned off" by sustained Gq-protein signaling in vivo

Diphtheria-toxin-mediated ablation of D1R-MSNs and D2R-MSNs recapitulates the behavioral phenotype of sustained Gq-protein signaling

To evaluate whether the observed changes elicited by sustained Gq-protein signaling in vivo are due to the dysfunction of MSNs, we first analyzed the immunoreactivity of the MSN marker DARPP-32 in D1R-Cre and D2R-Cre mice expressing hM3Dq-mCherry in the dorsal striatum. Chronic CNO treatment (10 mg/kg/d for 2 consecutive weeks) decreased DARPP-32 immunoreactivity similarly in D1R-Cre mice (relative value of CNO vs vehicle: 62 ± 6%; n = 8 –9 animals per group; $p < 0.01$) and D2R-Cre mice (relative value of CNO vs vehicle: 70 ± 5%; n = 7–8 animals per group; $p < 0.01$). This regime of CNO administration had no significant effect on wild-type mice injected with CAG-DIO-hM3Dq-mCherry-rAAV (relative value of CNO vs vehicle: 96 ± 9; n = 7–9 animals per group). Next, we evaluated the behavioral phenotype of D1R-Cre and D2R-Cre mice that had been injected stereotaxically into the dorsal striatum with a FLEX-rAAV encoding diphtheria toxin, which is well known to produce cell-population-specific ablation (Kreitzer & Berke 2011; Durieux et al. 2012; Kim et al. 2014). We selected three behavioral tests that rely at least in part on the dorsal striatum: the open field (to assess exploratory activity), Rotarod (to assess motor coordination), and Y-maze (to assess short-term spatial memory), and compared the phenotype of chronic CNO-treated hM3Dq-mCherry-expressing mice (Fig. 3A) with that of diphtheria-toxin-expressing mice (Fig. 3 E, F). Overall, the disrupting effects evoked by the selective expression of diphtheria toxin in D1R-MSNs or D2R-MSNs recapitulated very closely the respective changes elicited by sustained Gq signaling in the two MSN populations. Specifically, aside from the exploration assays, in which either chemogenetically or diphtheria toxin-induced dysfunction of D2R-MSNs enhanced locomotor activity (Fig.3 B, G), we found that either chemogenetically or diphtheria- toxin- induced dysfunction of D1R-MSNs impaired motor coordination (Fig. 3C, H), whereas either chemogenetically or diphtheria-toxin-induced dysfunction of D2R-MSNs impaired short-term spatial memory (Fig. 3 D, I; no differences in total arm entries were found among the different animal groups tested; data not shown).

The lack of motor coordination deficits shown by our D2R-Cre mice upon chronic CNO administration (Fig. 3C), compared with the data reported by Durieux et al. (2012) on mice in which A2AR-expressing neurons had

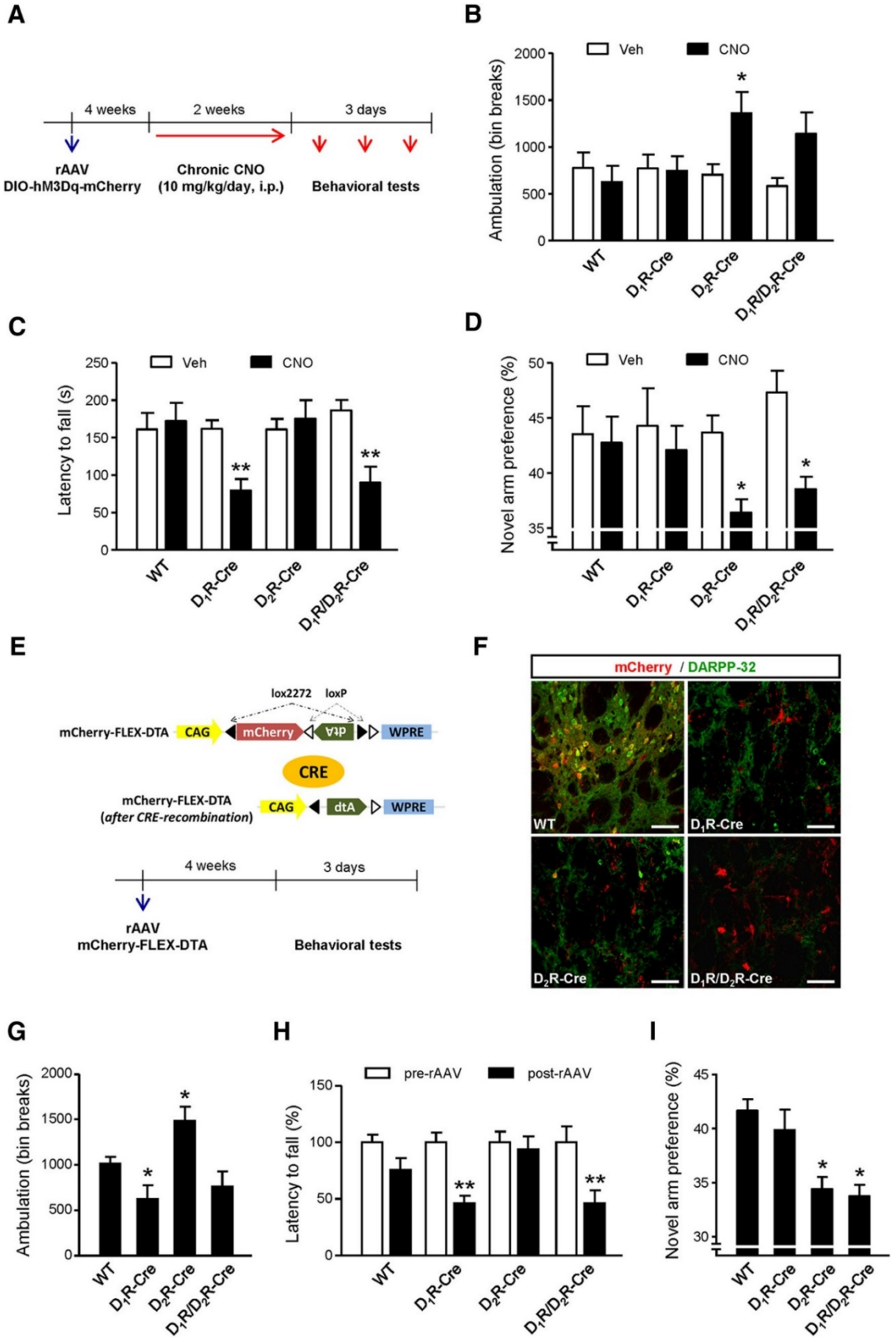

Figure 3. Diphtheria-toxin-mediated ablation of D1R-MSNs and D2R-MSNs recapitulates the behavioral phenotype of sustained Gq-protein signaling. A–D, Wild-type, D1R-Cre, D2R-Cre, and D1R/D2R-Cre mice were injected stereotaxically into the dorsal striatum with CAG-DIO-hM3Dq-mCherry-rAAV and left untreated for 4 weeks. Animals subsequently received one daily intraperitoneal injection of vehicle (saline) or CNO (10 mg/kg) for 2 consecutive weeks. Shown is the scheme of the chronic CNO treatment experiment. The arrows indicate injections (blue, rAAV; red, vehicle/CNO; A). Effect of chronic CNO treatment on ambulation (open field; B), motor coordination (Rotarod; C), and spatial recognition (Y-maze; D). E–I, Wild-type, D1R-Cre, D2R-Cre, and D1R/D2R-Cre mice were injected stereotaxically into the dorsal striatum with a rAAV encoding mCherry-FLEX-DTA and left untreated for 4 weeks. Scheme of the diphtheria toxin expression experiment. The blue arrow indicates rAAV injections (E). Fluorescence labeling of mCherry and DARPP-32 shows the effect of Cre-driven recombination in the mCherry-FLEX-DTA animals. Scale bar, 50 µm (F). Effect of diphtheria toxin expression on ambulation (open field; G), motor coordination (Rotarod; H), and spatial recognition (Y-maze; I). *p<0.05, **p<0.01 from the corresponding vehicle group (B–D), wild-type group (G, I), or pre-rAAV group (H). DTA, Diphtheria toxin fragment A.

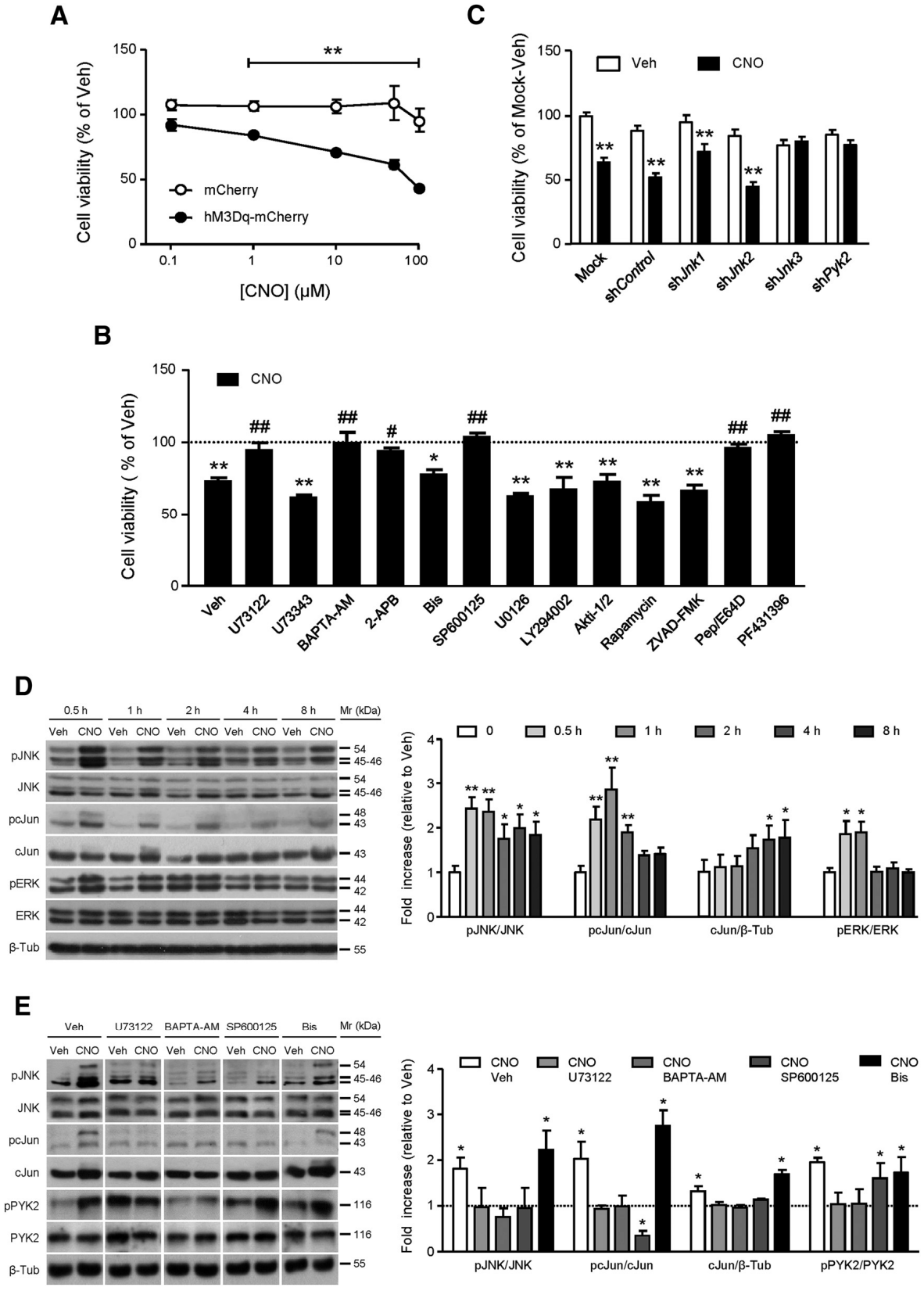

Figure 4. Sustained Gq-protein signaling induces the death of STHdh cells via PLC/Ca 2+/PYK2/JNK. STHdh cells were nucleofected with constructs expressing hM3Dq-mCherry or mCherry. A, Cells were incubated for 8 h with vehicle or the indicated doses of CNO. B, Cells were incubated for 8 h with vehicle or 50 μM CNO, together with vehicle or the indicated additions (see experimental details in Table 1). C, Cells were also nucleofected with a nontargeted shRNA, or with shRNAs directed against Jnk1, Jnk2, Jnk3, or Pyk2 and subsequently incubated for 8 h with vehicle or 50 μM CNO. Relative cell viability is shown in all panels. D, STHdh cells were incubated for the times indicated with vehicle or 50 μM CNO. E, STHdh cells were incubated for 2 h with vehicle or 50 μM CNO, together with vehicle or the indicated additions (see experimental details in Table 1). In D and E, the representative Western blots with the molecular weight of the protein bands (left) and quantification of optical density values of the protein bands relative to those of loading controls (right) are shown. In E (left), images from different parts of the same gel or from different gels were grouped. *p < 0.05, **p < 0.01 from the corresponding vehicle-treated cells. #p < 0.05, ##p < 0.01 from the corresponding CNO-vehicle-treated cells. Bis, Bisindolylmaleimide; Pep, pepstatin A.

been ablated, could be due to the different experimental conditions used. Therefore, we only analyzed Rotarod performance after training, whereas they found a Rotarod impairment only at the beginning of the training period, after which animals reached the same values as controls. Their D1R-MSN-ablated mice showed, like ours, a persistent Rotarod impairment also at the end of the training period (Durieux et al. 2012).

Together, these observations suggest that sustained Gq-protein signaling might impair striatal circuits in vivo by inducing the inactivation of direct-pathway and indirect-pathway MSNs.

Sustained Gq-protein signaling induces the death of MSNs via a PLC/Ca $^{2+}$/PYK2/JNK pathway

To analyze the molecular mechanism of Gq-driven action on MSNs, we first used cultures of STHdh mouse striatal neuroblasts, a well-established MSN-like cell model (Trettel et al. 2000). Cells were electroporated with a plasmid encoding hM3Dq-mCherry (or only mCherry) and subsequently treated with CNO (or vehicle). Exposure of cells expressing hM3Dq-mCherry (but not mCherry) to CNO decreased viability in a dose- dependent manner (Fig. 4A). From these assays, a dose of 50 µM CNO was selected for further experiments aimed at deciphering the signal transduction pathways responsible for Gq-driven cell death (Table 1). The phospholipase C (PLC) inhibitor U73122 (but not its inactive analog U73343), the intracellular Ca^{2+} chelator BAPTA-AM, and the intracellular Ca^{2+}-release inhibitor 2-APB prevented Gq-evoked cell death (Fig. 4B), thus supporting the involvement of PLC/Ca^{2+} signaling. In contrast, the general protein kinase C (PKC) inhibitor bisindolylmaleimide was ineffective. When assessing potential downstream effectors of PLC/Ca^{2+}, we found that the cJun *N*-terminal kinase (JNK) inhibitor SP600125 prevented Gq-induced cell death, whereas blockade of the extracellular signal- regulated kinase (ERK) cascade (with the MEK inhibitor U0126), phosphatidylinositol 3-kinase (PI3K; with LY294002), Akt (with Akti-1/2), or mammalian target of rapamycin complex 1 (mTORC1; with rapamycin) did not affect Gq action (Fig. 4B). Gq-driven STHdh cell death was caspase independent (as evidenced by the lack of effect of the pan-caspase inhibitor ZVAD-FMK), but lysosome dependent (as inferred from the preventive effect of the lysosomal-protease/cathepsin inhibitors pepstatin A and E64d; Fig. 4B).

To further clarify the involvement of the JNK cascade in cell death, we conducted additional experiments in STHdh cells. First, Gq-evoked cell death was prevented by a shRNA targeting JNK3, the most

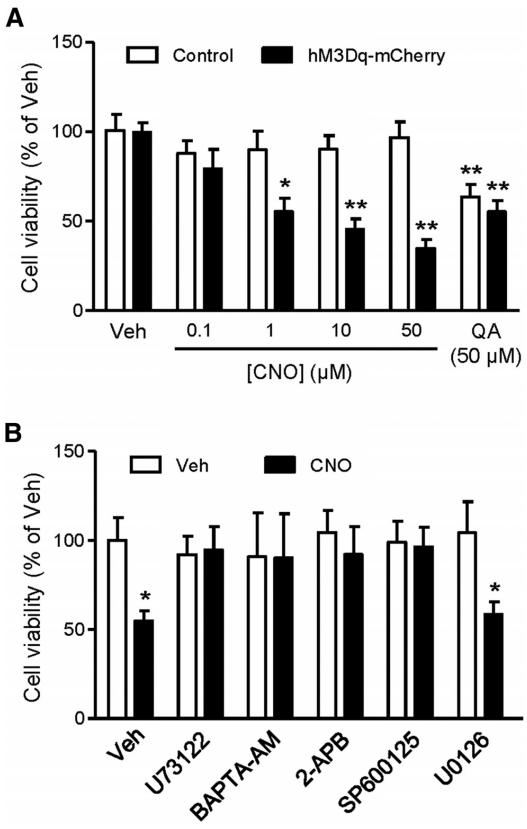

Figure 5. Sustained Gq-protein signaling induces the death of striatal neurons. A, Primary mouse striatal neurons were infected with CAG-hM3Dq-mCherry-rAAV or control CAG-GFP- rAAV at day 2 in vitro. At day 13 in vitro, they were incubated for 8 h with the indicated doses of CNO. Quinolinic acid (QA) was used as a control neurotoxin to demonstrate a comparable sen- sitivity to death of CAG-hM3Dq-mCherry-rAAV-infected neurons and CAG-GFP-rAAV-infected neurons. B, Primary mouse striatal neurons were infected with hM3Dq-mCherry-rAAV at day 2 in vitro. At day 13 in vitro, they were incubated for 8 h with vehicle or 50 µM CNO, together with vehicle or the indicated additions (see experimental details in Table 1). *p < 0.05, **p < 0.01 from the corresponding vehicle-treated cells.

relevant of the three JNK family members in the brain (Fig. 4C).

Second, Western blot experiments showed that activation of Gq signaling led to a sustained (up to 8 h) phosphorylation (activation) of JNK, which was accompanied by a phosphorylation (activation) and stabilization (increased levels) of its canonical substrate, the transcription factor cJun (Fig. 4D). In contrast, the phosphorylation (activation) of ERK, which was used as a control pathway triggered by acute Gq-evoked activation (Girault 2012), was only transient and returned to basal levels after2h of CNO challenge (Fig. 4D). Third, consistent with the aforementioned cell death experiments, the sustained Gq-evoked activation of JNK and cJun was PLC/Ca^{2+} dependent (as shown by the preventive effect of U73122 and BAPTA-AM) and PKC independent (as shown by the lack of effect of bisindolylmaleimide; Fig. 4E).

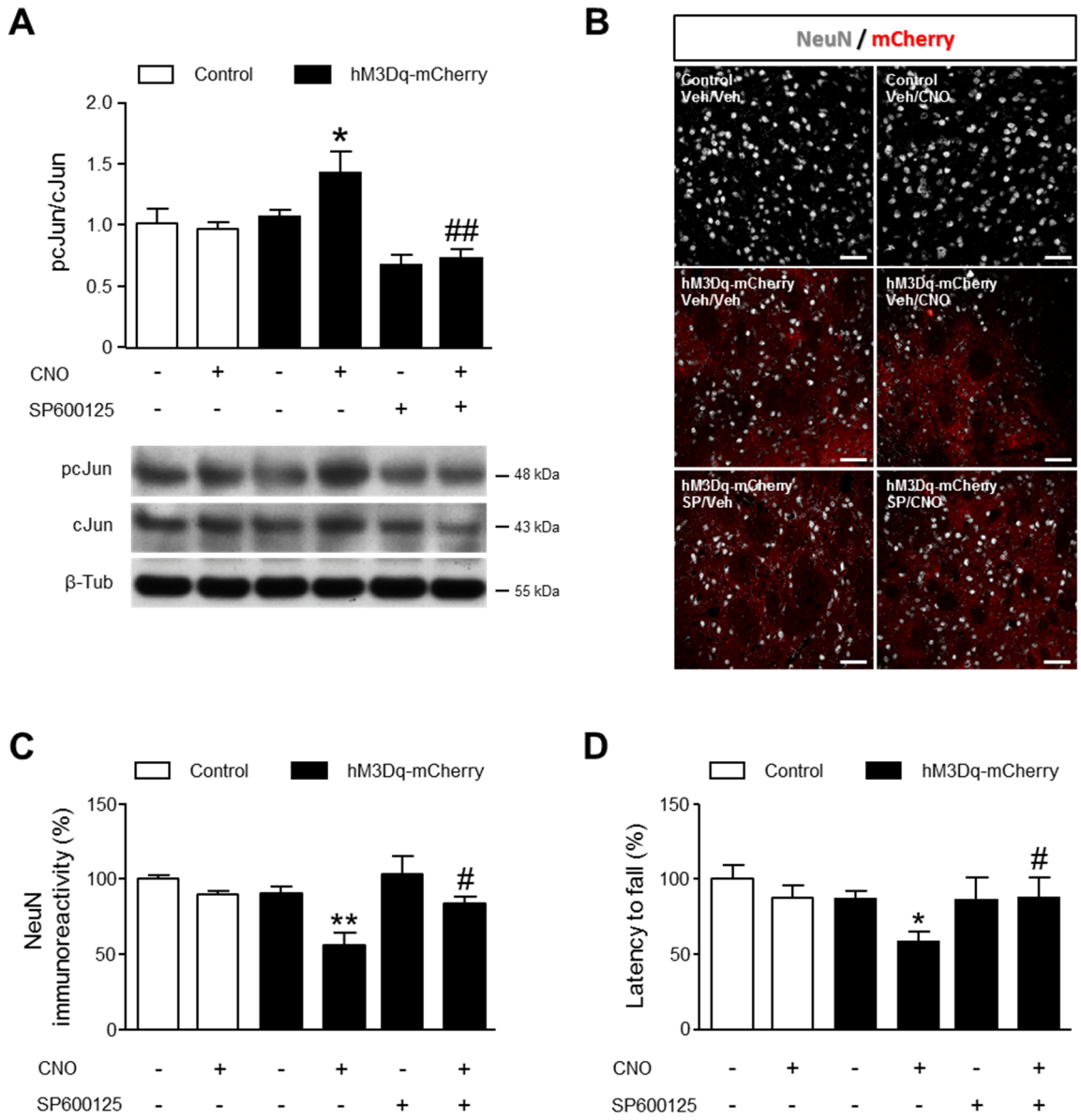

Figure 6. Sustained Gq-protein signaling disrupts the functionality of striatal neurons in vivo via JNK. C57BL/6N mice were injected stereotaxically into the dorsal striatum with CAG-hM3Dq-mCherry-rAAV or control CAG-rAAV and left untreated for 4 weeks. A, Animals subsequently received a single intraperitoneal injection of vehicle or CNO (10 mg/kg), together with a single intraperitoneal injection of vehicle or SP600125 (15 mg/kg), and, after 2 h, their dorsal striata were excised for Western blot analysis. Quantification of optical density values of phosphorylated cJun relative to those of the loading control (total cJun; top) and representative Western blots (bottom) are shown. B–D, Animals subsequently received one daily intraperitoneal injection of vehicle or CNO (10 mg/kg), together with one daily intraperitoneal injection of vehicle or SP600125 (15 mg/kg), for 10 consecutive days. Shown are representative images of NeuN immunostaining. Scale bar, 50 µm (B). Shown is the quantification of NeuN expression (relative values of NeuN-positive cells; C) and Rotarod test performance (latency to fall; D). *p < 0.05, **p < 0.01 from the corresponding vehicle-vehicle group; #p < 0.05, ##p < 0.01 from the corresponding CNO-vehicle group.

We next investigated the link between Ca^{2+} and JNK. Proline-rich tyrosine kinase 2 (PYK2) is a cytoplasmic non-receptor tyrosine kinase enriched in neurons that controls various neurobiological functions and that, by acting as a Ca^{2+} effector, can activate mitogen-activated protein kinase cascades (Girault et al. 1999). Therefore, we investigated whether PYK2 was involved in our experimental setting. The Gq-evoked death of STHdh cells was prevented by the dual PYK2/focal adhesion kinase inhibitor PF431396 (Fig. 4B), as well as by a Pyk2-directed shRNA (Fig. 4C). Likewise, activation of Gq signaling led to the phosphorylation (activation) of PYK2 and this effect was prevented by U73122 and BAPTA-AM, but not by SP600125 (Fig. 3E), thus supporting that PYK2 is downstream of PLC/Ca^{2+} and upstream of JNK.

We subsequently investigated whether the Gq-triggered effects observed in STHdh cells could be extrapolated to a more physiological experimental model as primary striatal neurons. Indeed, activation of Gq signaling upon challenge of hM3Dq-mCherry expressing primary mouse striatal neurons to CNO also led to a PLC/Ca^{2+}/JNK-dependent, ERK-independent cell death process (Fig. 5 A, B).

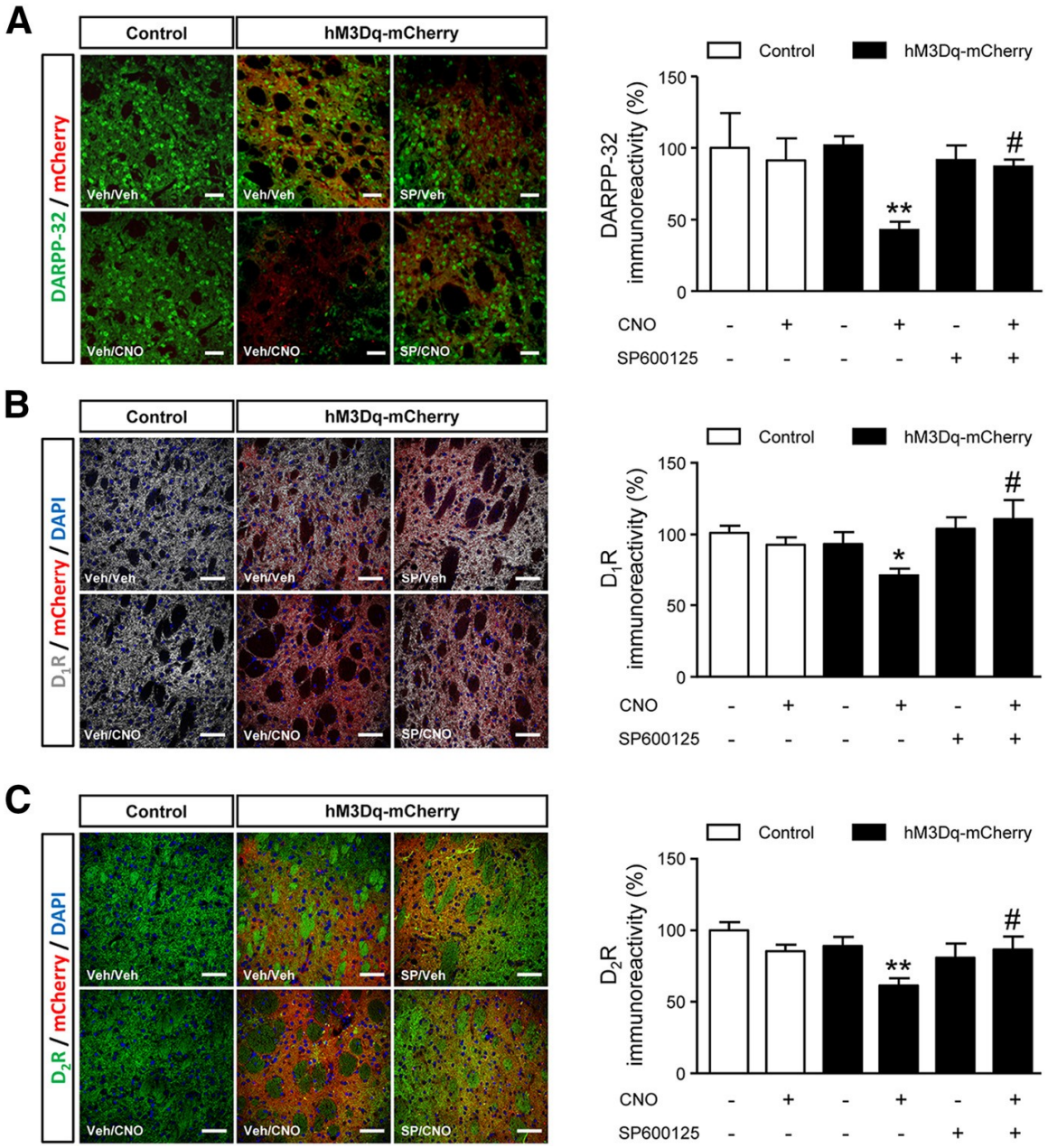

Figure 7. Sustained Gq-protein signaling induces the loss of MSNs in vivo via JNK. C57BL/6N mice were injected stereotaxically into the dorsal striatum with CAG-hM3Dq-mCherry-rAAV or control CAG-rAAV, and left untreated for 4 weeks. Animals subsequently received one daily intraperitoneal injection of vehicle or CNO (10 mg/kg), together with one daily intraperitoneal injection of vehicle or SP600125 (15 mg/kg), for 10 consecutive days. A, DARPP-32 expression (relative values of DARPP-32 immunoreactivity). B, D1R expression (relative values of D1R immunoreactivity). C, D2R expression (relative values of D2R immunoreactivity). Representative images are shown in all panels. Scale bar, 50µm. *p < 0.05, **p < 0.01 from the corresponding vehicle/vehicle group; # p < 0.05 from the corresponding CNO/vehicle group.

In sum, these data show that sustained Gq-protein activation signals neuronal cell death via a PLC/Ca^{2+}/PYK2/JNK-dependent pathway.

Sustained Gq-protein signaling disrupts the functionality of D1R-MSNs and D2R-MSNs in vivo via JNK

To evaluate the functional relevance of JNK in vivo, we first injected C57BL/6N mice stereotaxically into the dorsal striatum with a rAAV encoding hM3Dq-mCherry (or control rAAV). The transgene was driven by the CAG promoter to allow its expression in all MSNs. In agreement with our aforementioned cell culture observations, engagement of Gq signaling (one single intraperitoneal injection of CNO at 10 mg/kg in hM3Dq-mCherry-expressing mice) triggered striatal JNK activation in vivo, as determined by the SP600125-sensitive phosphorylation (activation) of cJun (Fig. 6A; we were unable to obtain technically reliable Western blots from mouse striatal extracts with commercial anti-pJNK antibodies).

Furthermore, after sustained Gq signaling (one daily intra-peritoneal injection of CNO at 10 mg/kg for 10 d), we found a loss of MSNs, as determined by the

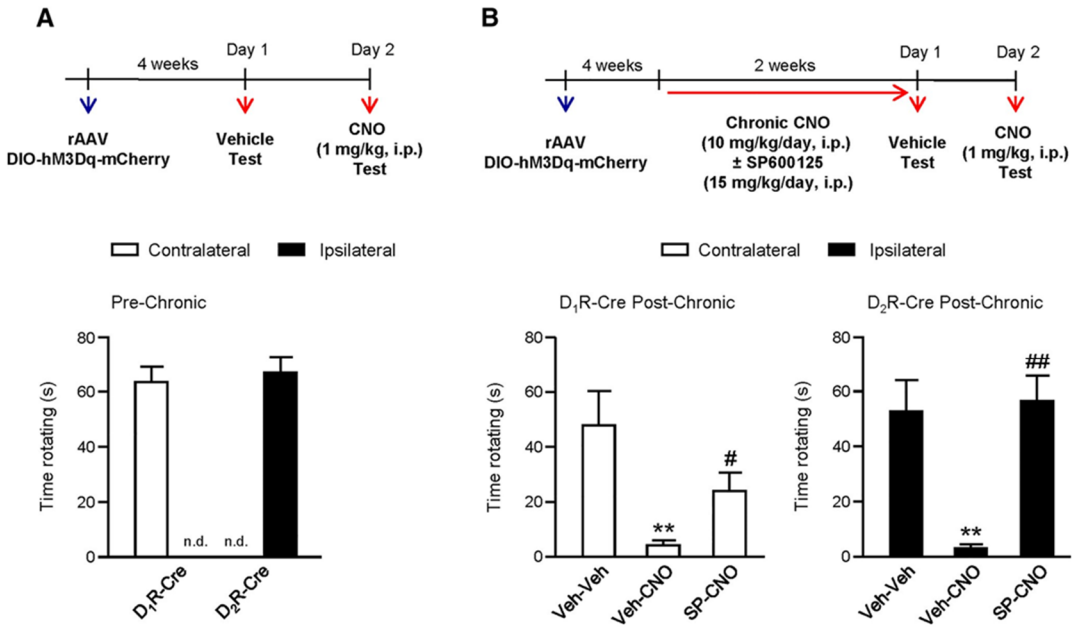

Figure 8. Sustained Gq-protein signaling disrupts the functionality of D1R-MSNs and D2R-MSNs in vivo via JNK. Wild-type, D1R-Cre, and D2R-Cre mice received a unilateral injection into the right dorsal striatum of CAG-DIO-hM3Dq-mCherry-rAAV and were left untreated for 4 weeks. A, Animals subsequently received a single injection of vehicle or, after 24 h, of CNO (1 mg/kg); in both cases, animals were tested 1 h later in an open field. A scheme of this acute CNO treatment experiment is shown on top. The arrows indicate injections (blue, rAAV; red, vehicle/CNO). B, Animals subsequently received one daily intraperitoneal injection of vehicle or CNO (10 mg/kg), together with one daily intraperitoneal injection of vehicle or SP600125 (15 mg/kg), for 15 consecutive days. One day after the last treatment, all mice were injected with vehicle and, after 24 h, with CNO (1 mg/kg); in both cases, animals were tested 1 h later in an open field (left, D1R-Cre mice; right, D2R-Cre mice). A scheme of this chronic CNO treatment experiment is shown at the top. The arrows indicate injections (blue, rAAV; red, vehicle/CNO). Ipsilateral and contralateral movements were monitored for 5 minutes. **p < 0.01 from the corresponding vehicle/vehicle group. #p < 0.05, ##p < 0.01 from the corresponding vehicle-CNO group. See details of statistical analyses in Table 2. n.d., Nondetectable.

reduction of the neuronal marker NeuN (Fig. 6 B, C) and the MSN marker DARPP-32 (Fig. 7A). This loss of MSNs was equally evident in the D1R-MSN population (Fig. 7B) and the D2R-MSN population (Fig. 7C). Moreover, these alterations in neuronal markers were accompanied by a deficit in the Rotarod test (Fig. 6D), a well-established behavioral readout of the dorsal striatum. Remarkably, these Gq-evoked effects were prevented by pharmacological blockade of JNK (Figs.6B–D, 7A–C) and were not simply caused by the viral expression of a novel receptor or by an off-target action of CNO (either hM3Dq-mCherry expression in the absence of CNO or treatment of control rAAV-infected animals with CNO was ineffective; Figs. 6A–D, 7A–C).

Finally, we assessed whether JNK-driven signaling affects either the direct pathway or the indirect pathway separately by monitoring a clear-cut behavioral task as contraversive movements (Tecuapetla et al. 2014). Selective acute unilateral activation of Gq signaling in D1R-MSNs or D2R-MSNs induced contralateral or ipsilateral movements, respectively (Fig. 8A). This acute effect was abrogated in those animals that had been chronically treated with CNO (Fig. 8B). In turn, this impairing effect of chronic CNO treatment on contralateral/D1R-MSN-dependent movements (Fig. 8B, left) and ipsilateral / D2R-MSN-dependent movements (Fig. 8B, right) was rescued by the coadministration of a JNK inhibitor to the animals. Collectively, these data show that JNK mediates the inactivation of MSNs and the disruption of striatal circuits evoked by sustained Gq-protein signaling in vivo.

CHAPTER 2

The vast majority of striatal neurons are GABAergic medium spiny neurons (MSNs). These cells receive glutamatergic input primarily from the cortex, and form two major efferent pathways: the direct (striatonigral) pathway, expressing dopamine D1 receptor (D1R-MSNs), and the indirect (striatopallidal) pathway, expressing dopamine D2 receptor (D2R-MSNs). Different mechanisms that lead to the degeneration of MSNs have been defined in preclinical models of striatal damage. However, a key unanswered question is which precise molecular factors may dictate a selective susceptibility of D1R-MSNs and D2R-MSNs in archetypical striatal neurodegenerative diseases such as Huntington's disease (HD). In this chapter, by using an array of genetic, chemogenetic and pharmacological strategies to manipulate cannabinoid CB1R function in a spatiotemporally-restricted manner in vivo, we show that CB1R located on corticostriatal projections, by blunting glutamatergic output, selectively safeguards D1R-MSNs of the mouse dorsal striatum from cortical mtHtt-induced damage. Specifically, expression of mtHtt in the motor cortex damages D1R-MSNs but not D2R-MSNs upon (i) pharmacological blockade of CB1R with rimonabant or (ii) conditional genetic deletion of CB1R in cortical principal neurons. This neurotoxic process is rescued by administration of the NMDA receptor antagonist MK-801. Likewise, a selective vulnerability of D1R-MSNs vs. D2R-MSNs is observed when corticostriatal glutamatergic projections are overactivated remotely by means of a DREADD pharmacogenetics approach and CB1R is pharmacologically blocked. CB1R located on corticostriatal projections, by inhibiting glutamatergic transmission, also protects MSNs from mtHtt-expressing astroglia. Finally, astroglial MGL controls the availability of the 2-AG to ensure protection of MSNs. Taken together, these findings define cortical CB1R as a key neurochemical player in dictating a dissimilar vulnerability of D2R-MSNs vs. D1R-MSNs, and may contribute to understand the role of coordinated cannabinergic-glutamatergic signaling in the control of the corticostriatal direct pathway and its dysregulation in HD.

CAPÍTULO 2

La gran mayoría de las neuronas estriatales son neuronas GABAérgicas espinosas medianas (MSNs del inglés medium spiny neurons). Estas células reciben aferencias glutamatérgicas principalmente de la corteza y forman dos vías eferentes principales: la vía directa (estriatonigral), que está fomada por MSNs que expresan el receptor de dopamina D1 (D1R-MSNs) y la vía indirecta (striatopallidal), formada por MSNs que expresan el receptor de dopamina D2 (D2R-MSNs).Se han descrito diferentes mecanismos que conducen a la degeneración de los MSNs en modelos preclínicos de daño estriatal. Sin embargo, una de las preguntas que aún no se han resuelto es qué factores moleculares específicos pueden dictar una susceptibilidad selectiva entre D1R-MSNs y D2R-MSNs en enfermedades neurodegenerativas estriatales arquetípicas, como la enfermedad de Huntington (EH). En este capítulo, mediante estrategias genéticas, quimiogenéticas y farmacológicas para manipular la función de CB1R de manera espaciotemporalmente restringida *in vivo*, se muestra que el CB1R situado en las proyecciones corticoestriatal, reduciendo la señalización glutamatérgica, protege selectivamente las D1R-MSNs estriatales frente al daño derivado de la expresión de mtHtt en corteza. En concreto, la expresión de mtHtt en corteza daña las D1R-MSNs pero no las D2R-MSNs en el contexto de (i) bloqueo farmacológico de CB1R con rimonabant o (ii) delección condicional de CB1R en neuronas corticales principales. Este efecto neurotóxico se previene con la administración del antagonista del receptor NMDA MK-801. Asimismo, se observa una vulnerabilidad selectiva de D1R-MSNs y D2R-MSNs cuando se sobreactivan de forma remota las proyeccioes corticoestriatales glutamatérgicas, mediante una aproximación farmacogenética con DREADD, mientras CB1R está bloqueado farmacológicamente. El CB1R situado en las proyecciones corticoestriatales, mediante inhibición de la transmisión glutamatérgica, también protege las MSNs del daño derivado por la expresión de mHtt en astrocitos. Por último, la MGL astroglial controla la disponibilidad del 2-AG, participando así en la protección de las MSNs. Así, el CB1R cortical representa un factor neuroquímico clave para determinar la diferente vulnerabilidad entre D1R-MSNs y D2R-MSNs, pudiendo contribuir a entender la coordinación entre señalización cannabinérgica-glutamatérgica en el control de la vía directa corticoestriatal y su alteración en la EH.

RESULTS-OBJECTIVE 2

D1R-MSNs and D2R-MSNs are equally vulnerable to cell-autonomous mutant huntingtin-induced damage

To evaluate the potentially different vulnerability of D1R-MSNs and D2R-MSNs to huntingtin-induced degeneration we first used a rAAV-vector delivery strategy based on the expression of CFP-tagged human huntingtin exon 1 harboring a pathogenic polyQ tract of 94 CAG repeats (herein used as a model of mutant huntingtin, mtHtt) or a normal, non-pathogenic polyQ tract of 16 CAG repeats (herein used as a model of wild-type huntingtin, wtHtt). The expression of the transgene was driven by a minimal CaMKIIα promoter in order to confine it to the main cell populations affected in HD, namely MSNs (when viral injections were performed into the dorsal striatum) and principal cortical neurons (when viral injections were performed into the motor cortex). Viral inoculation was conducted in BAC transgenic mice expressing the tdTomato and EGFP reporter genes under the control of the D1R and D2R promoter, respectively (Fig. 1B).

When selectively expressed in MSNs of the dorsal striatum, mtHtt produced, 2 weeks after viral injection, a remarkable loss of the *pan*-MSN marker dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32), as well as a parallel reduction of cells that were positive for tdTomato fluorescence (*i.e.*, D1R-MSNs) or EGFP fluorescence (*i.e.*, D2R-MSNs) (Fig. 1C). This was further corroborated by the comparable mtHtt-induced decrease of D1R and D2R staining that was found when immunofluorescence studies were conducted with antibodies raised against each of the two receptors (Fig. 1C). Of note, D1R-MSNs and D2R-MSNs were equally vulnerable to mtHtt-induced damage (Fig. 1C). Moreover, this loss of MSNs had a notable functional impact as evidenced by the impairment of Rotarod performance, a well-established motor coordination paradigm that relies, at least large part, on striatal function (Fig. 1E). In contrast to this damaging effect of mtHtt expressed by MSNs *in situ*, no significant loss of neuronal markers or decline in RotaRod performance was observed when mtHtt was selectively expressed in principal neurons of the motor cortex, even 4 weeks after viral injection (Fig. 1D-E).

Taken together, these data indicate that D1R-MSNs and D2R-MSNs are equally sensitive to cell-autonomous mtHtt-induced toxicity, and that expression of mtHtt in cortical principal neurons is not sufficient *per se* to produce a significant damage of MSNs, at least under these experimental conditions.

Expression of mutant huntingtin in the cortex damages D1R-MSNs but not D2R-MSNs upon CB1R pharmacological blockade

CB1R, one of the most abundant metabotropic receptors in the brain, is highly expressed in both MSNs and corticostriatal projections (Katona & Freund 2008), plays a key role in the control of motor behavior (Castillo et al. 2012), and protects MSNs in animal models of excitotoxicity (Fernández-Ruiz et al. 2011) and HD (Blázquez et al. 2011; Mievis et al. 2011; Chiarlone et al. 2014). However, it is not known whether CB1R-evoked neuroprotection may be selective for different MSN populations, and, if so, which precise population(s) of CB1R molecules would be involved in such effect. To evaluate this question we first injected -as above- CaMKIIα promoter-driven wtHtt or mtHtt-expressing rAAV vectors into the dorsal striatum or the motor cortex of D1R-tdTomato/D2R-EGFP reporter mice, and subsequently treated them with vehicle or the CB1R-selective antagonist SR141716 (rimonabant) at 1 mg/kg/d (i.p.) for 2 weeks (viral inoculation into the striatum) or 4 weeks (viral inoculation into the cortex) (Fig 2A). Rimonabant did not affect striatum-autonomous mtHtt-mediated loss of D1R-MSN or D2R-MSN markers, nor worsened motor coordination deficits under these conditions (Fig. 2B,C). In remarkable contrast, CB1R pharmacological blockade worsened these hallmarks of striatal integrity when mtHtt was selectively expressed in principal neurons of the motor cortex (Fig. 2B,C). Moreover, MSN damage under these conditions exclusively entailed D1R-MSNs, while D2R-MSNs remained unaffected (Fig. 2B).

Taken together, these observations support that, when CB1R activity is compromised, expression of mtHtt in cortical principal neurons determines a dissimilar susceptibility of D1R-MSNs vs. D2R-MSNs to damage.

CB1R located on corticostriatal projections protects D1R-MSNs but not D2R-MSNs from cortex-elicited damage

To test the aforementioned hypothesis more cogently we used three different experimental paradigms in which cortical CB1R function and neuronal activity were manipulated *in vivo* in a spatiotemporally-selective manner.

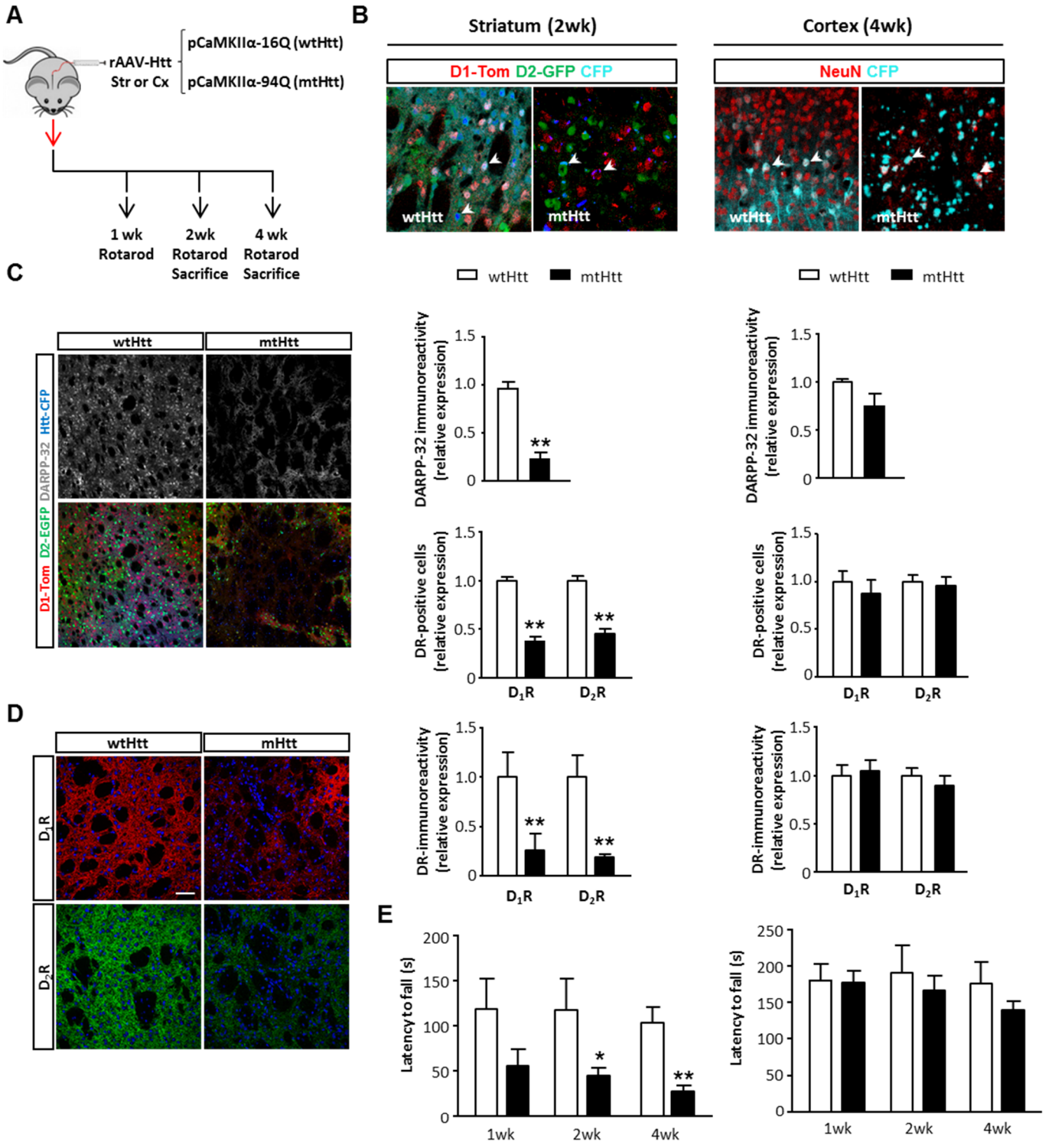

Figure 1. D1R-MSNs and D2R-MSNs are equally vulnerable to cell-autonomous mutant huntingtin-induced damage. *Drd1a*-tdTomato/*Drd2*-EGFP mice were injected stereotactically into the dorsal striatum or the motor cortex with rAAV vectors encoding wtHtt (16Q-CFP) or mtHtt (94Q-CFP) under the control of a CaMKIIα promoter. A, Scheme of the experiment. B, Expression of the vectors. C, DARPP-32 immunoreactivity, tdTomato (D1R) and EGFP (D2R) positive cells in the dorsal striatum. D, D1R and D2R immunoreactivity in the dorsal striatum. In C-D, data are expressed as relative values of the wtHtt group, and correspond to 1-2 weeks (striatum) or 4 weeks (cortex) after viral inoculation. Representative images are shown. Scale bar, 50 µm. E, Rotarod performance (time to fall), data are presented as mean ± SEM. *p<0.05, **p<0.01 from the corresponding wtHtt by unpaired t-test (n = 6-8) (C-D), or two-way ANOVA (n = 4-8) (E).

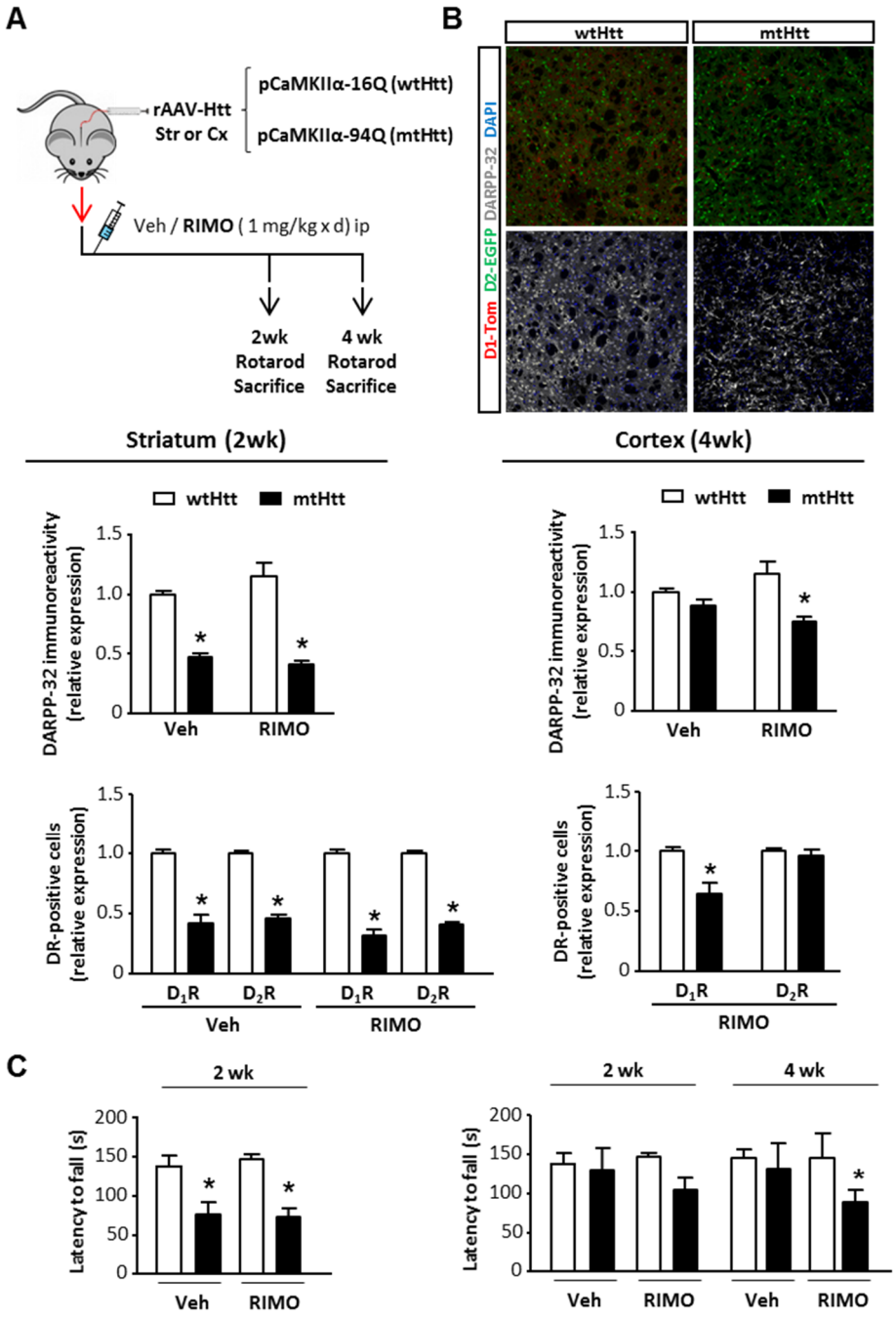

Figure 2. Expression of mutant huntingtin in the cortex damages D1R-MSNs but not D2R-MSNs upon CB1R pharmacological blockade. *Drd1a*-tdTomato/*Drd2*-EGFP mice were injected stereotactically into the dorsal striatum or the motor cortex with rAAV vectors encoding wtHtt (16Q-CFP) or mtHtt (94Q-CFP) under the control of a CaMKIIα promoter. Animals were subsequently treated with vehicle or SR141716 (rimonabant; 1 mg/kg/d, i.p.) for 2 weeks (viral inoculation into the striatum) or 4 weeks (vital inoculation into the cortex). A, Scheme of the experiment. B, DARPP-32 immunoreactivity, tdTomato (D1R) and EGFP (D2R) positive cells in the dorsal striatum. Data are expressed as relative values of the wtHtt-vehicle group. Representative images are shown. Scale bar, 50 µm. C, Rotarod performance (time to fall), data are presented as mean ± SEM. *p<0.05, **p<0.01 from the corresponding wtHtt-vehicle group by unpaired t-test (n = 6-8).

In a first experimental paradigm, conditional mutant mice bearing a genetic deletion of CB1R in dorsal telencephalic glutamatergic neurons ($CB1R^{floxed/floxed;Nex\text{-}Cre/+}$ mice; herein referred to as Glu-$CB1R^{-/-}$ mice), and their $CB1R^{floxed/floxed}$ control littermates, were injected with CaMKIIα promoter-driven wtHtt or mtHtt-expressing rAAV vectors into the motor cortex (Fig. 3A). As above, no significant mtHtt-evoked striatal toxicity was observed in control mice (Fig. 3B,D). In contrast, cortical mtHtt induced a neurotoxic effect on the striatum of Glu-$CB1R^{-/-}$ mice, as determined by a loss of striatal DARPP-32 and postsynaptic density protein 95 (PSD-95) expression, as well as a decline in Rotarod performance (Fig. 3B,D). Remarkably, and in line with the rimonabant experiment on D1R-tdTomato/D2R-EGFP reporter

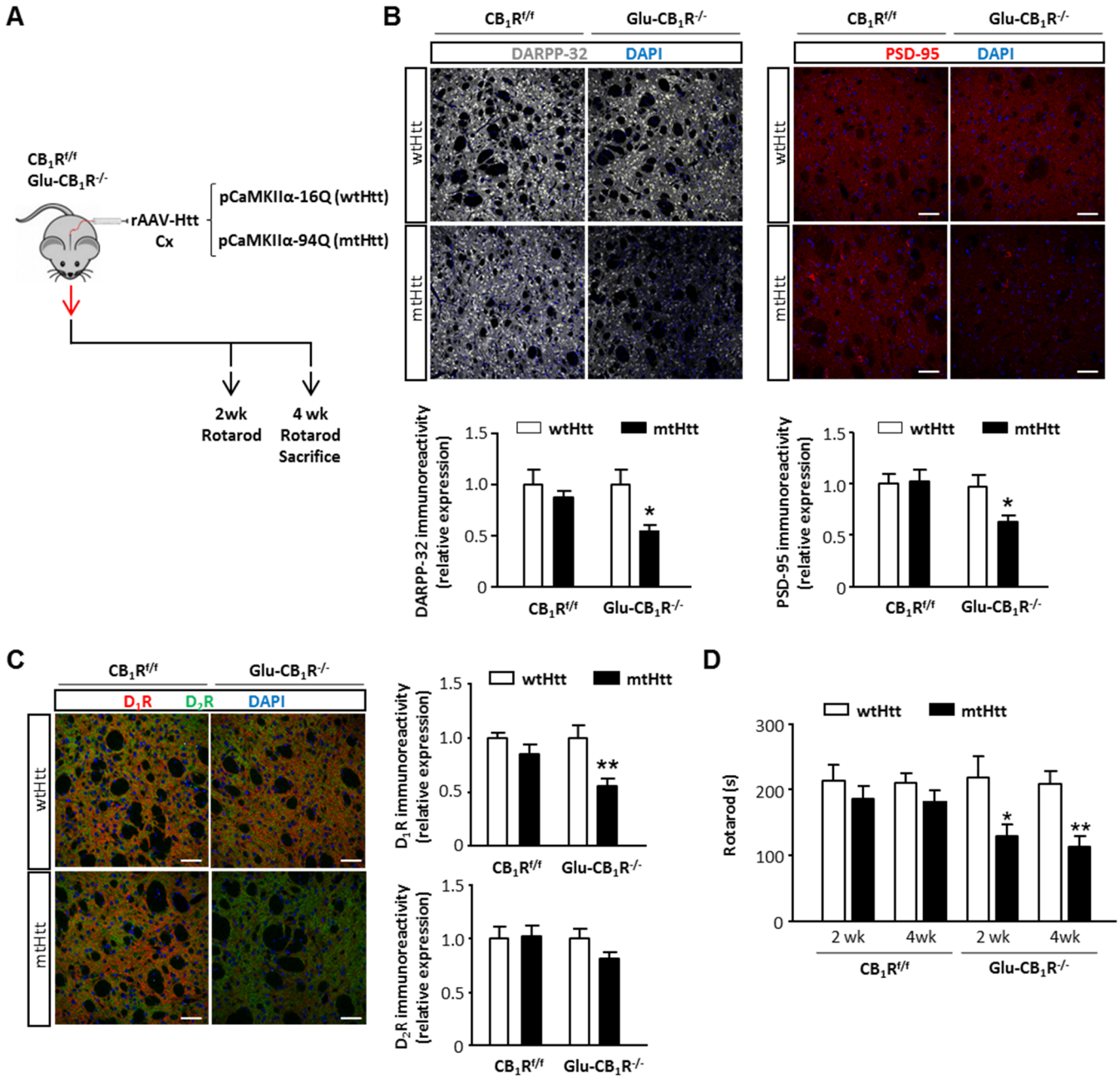

Figure 3. Genetic inactivation of cortical CB1R enhances the vulnerability of D1R-MSNs but not D2R-MSNs to cortical mutant huntingtin-elicited damage. Glu-CB1R$^{-/-}$ mice and CB1R$^{floxed/floxed}$ littermates were injected stereotactically into the motor cortex with rAAV vectors encoding wtHtt (16Q-CFP) or mtHtt (94Q-CFP) under the control of a CaMKIIα promoter. A, Scheme of the experiment. B, DARPP-32 and PSD-95 immunoreactivity in the dorsal striatum. C, D1R and D2R immunoreactivity in the dorsal striatum. In B-C, data are expressed as relative values of the correspondent wtHtt group. Representative images are shown. Scale bar, 50 µm. D, Rotarod performance (time to fall), data are presented as mean ± SEM. *p<0.05, **p<0.01 from the corresponding wtHtt group by two-way ANOVA (n = 8-10).

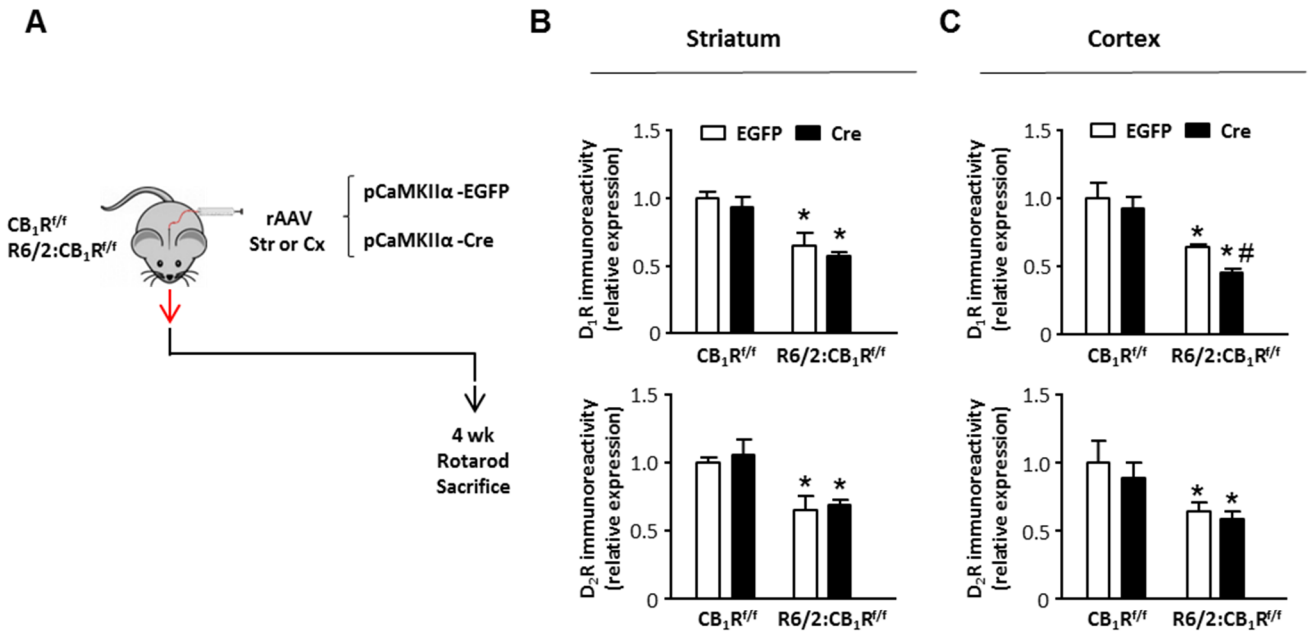

Figure 4. Genetic inactivation of cortical but not striatal CB1R enhances the vulnerability of D1R-MSNs but not D2R-MSNs in R6/2 mice. Four week-old R6/2L:CB1R$^{floxed/floxed}$ mice and CB1R$^{floxed/floxed}$ littermates were injected stereotactically into the motor cortex or the dorsal striatum with rAAV vectors encoding Cre recombinase or EGFP under the control of a CaMKIIα promoter as described (Chiarlone et. al. 2014). At week 20 of age, animals were sacrificed for histological analyses. A, Scheme of the experiment. B, D1R and D2R immunoreactivity in the dorsal striatum (upon viral inoculation into the striatum). C, D1R and D2R immunoreactivity in the dorsal striatum (upon viral inoculation into the cortex). In B-C, data are expressed as relative values of the EGFP-CB1R$^{floxed/floxed}$ group. *p<0.05, **p<0.01 from the corresponding CB1R$^{floxed/floxed}$ group. $^{\#}$p<0.05, $^{\#\#}$p<0.01 from the corresponding EGFP-R6/2L:CB1R$^{floxed/floxed}$ group by multiple t-test and Mann-Whitney test (n = 4-6).

mice described above, the impact of cortical mtHtt expression on Glu-CB1R$^{-/-}$ mice involved D1R-MSNs exclusively, while D2R-MSNs remained essentially intact (Fig. 3C).

In a second experimental paradigm, we crossed R6/2L mice, a transgenic model of HD that expresses in all body cells exon 1 of the human huntingtin gene with an largely expanded CAG tract, with CB1R$^{floxed/floxed}$ mice, thus generating a R6/2L:CB1R$^{floxed/floxed}$ line, that allows the spatiotemporally-controlled excision of the *loxP*-flanked CB1R gene by Cre recombinase (Chiarlone et al. 2014). These R6/2L:CB$_1$R$^{floxed/floxed}$ animals (and their CB1R$^{floxed/floxed}$ control littermates) were injected into the dorsal striatum or the motor cortex with a rAAV vector encoding Cre (or EGFP as control) under the control of the CaMKIIα promoter (Fig. 4A). We have previously shown that, under these conditions, CB1R gene inactivation in the motor cortex of R6/2L:CB1R$^{floxed/floxed}$ mice reduces striatal DARPP-32 expression and Rotarod performance (Chiarlone et al. 2014). As shown here in Fig. 4B, selective CB1R gene inactivation in dorsal-striatum MSNs did not affect D1R-MSNs and D2R-MSNs. In contrast, this procedure of selective CB1R gene inactivation in cortical principal neurons impacted D1R-MSNs selectively, while D2R-MSNs remained intact. (Fig. 4C).

In a third experimental paradigm, we sought to extrapolate the above observations on cortical mtHtt-induced striatal damage to another model of cortex-initiated striatal damage. Thus, we selectively enhanced neuronal activity with a designer receptor exclusively activated by designer drug (DREADD). This chemogenetic technique is based on the expression of engineered GPCRs that are selectively activated by systemically bioavailable, brain-penetrant and otherwise pharmacologically inert ligands such as clozapine-*N*-oxide (CNO) (Lee et al. 2014). Specifically, wild-type C57BL/6N mice were injected into the motor cortex with a rAAV vector encoding a Gq protein-coupled DREADD (hM3Dq) fused to mCherry under the control of the CaMKIIα promoter. Animals were subsequently treated with vehicle or CNO, in conditions known to evoke sustained cortical activation and thereby excitotoxic damage on the striatum (10 mg/kg/d, i.p., for 4 weeks) (Alexander et al. 2010; Chiarlone et al. 2014), as well as with vehicle or rimonabant (1 mg/kg/d, i.p.) (Fig.5A). As shown in Fig. 5B-D, CB1R pharmacological blockade sensitized D1R-MSNs but not D2R-MSNs to prolonged cortical overactivation.

Taken together, these findings support that CB1R located on corticostriatal projections protects D1R-MSNs but not D2R-MSNs from mtHtt or excitotoxicity-induced damage as derived from cortical principal neurons.

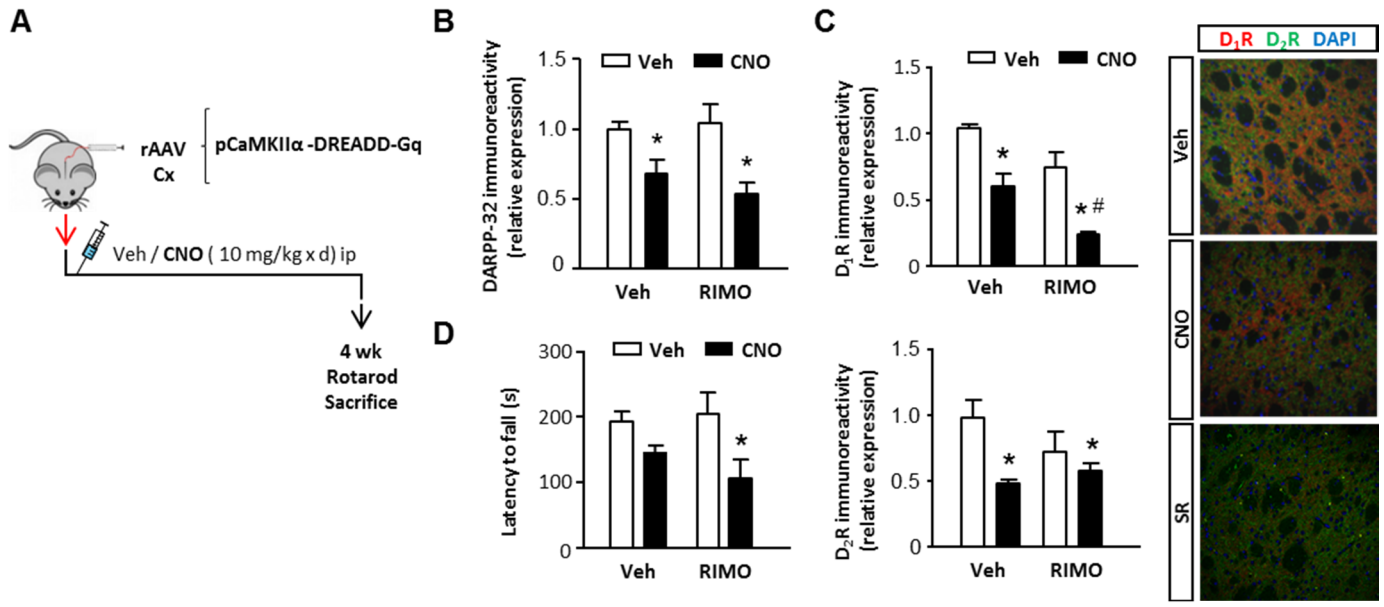

Figure 5. Sustained chemogenetic activation of cortical projections damages D1R-MSNs but not D2R-MSNs upon CB1R pharmacological blockade Eight week-old C57BL/6N mice were injected stereotactically into the motor cortex with a rAAV vector encoding hM3Dq-mCherry under the control of a CaMKIIα promoter as described (Chiarlone et. al. 2014). Six weeks later, animals were treated with vehicle or CNO (10 mg/kg/d, i.p), alone or in combination with vehicle or SR141716 (rimonabant; 1 mg/kg/day, i.p.), for 4 weeks. Rotarod performance was evaluated along the last 3 days of treatment, and the day after animals were sacrificed for histological analyses. A, Scheme of the experiment. B, DARPP-32 immunoreactivity in the dorsal striatum. C, D1R and D2R immunoreactivity in the dorsal striatum. In B-C, data are expressed as relative values of the vehicle-vehicle group. Representative images are shown. Scale bar, 50 µm. D, Rotarod performance (time to fall)), data are presented as mean ± SEM. *p<0.05, **p<0.01 from the vehicle-vehicle group. #p<0.05, ##p<0.01 from the vehicle-CNO group by two-way ANOVA (n = 3-6) .

CB_1R located on corticostriatal projections protects D1R-MSNs from cortex-elicited damage by inhibiting glutamatergic transmission

To evaluate whether cortical CB1R controls glutamatergic signaling onto the striatum we first measured by microdialysis extracellular glutamate concentration in the dorsal striatum of Glu-$CB1R^{-/-}$ mice and $CB1^{floxed/floxed}$ littermates. Neuronal depolarization with KCl produced a consecutive sequence of peaks of glutamate release that was higher in Glu-$CB1R^{-/-}$ mice than in control animals (Fig. 6A). As a control we analyzed in parallel GABA concentration and found no significant change upon cortical CB1R genetic ablation (Fig. 6A). Next, we isolated striatal synaptosomes from C57BL/6N mice and evaluated the effect of CB1R on glutamate release, thus assessing the activity of CB1R located on corticostriatal projections (Chiarlone et al. 2014). The cannabinoid receptor agonist HU-210 (5 µM) reduced KCl-evoked glutamate output from striatal synaptosomes. As a proof of specificity, and in agreement with the coupling of CB1R to Gi/o and G12/13 proteins in cortical principal neurons (Castillo et al. 2012; Roland et al. 2014), the effect of HU-210 on glutamate release was attenuated by pertussis toxin (which inactivates Gi/o) and CCG-1423 (which inhibits G12/13 protein/Rho-mediated signaling) (Fig. 6B).

To assess the functional impact of the CB1R receptor-mediated control of glutamatergic signaling on striatal integrity *in vivo* we injected Glu-$CB1R^{-/-}$ mice with CaMKIIα promoter-driven wtHtt or mtHtt-expressing vectors into the motor cortex, and treated them with vehicle or the NMDAR-selective antagonist MK-801 at 0.03 mg/kg/day (i.p.) for 4 weeks (Fig. 6C). MK-801 administration effectively rescued the loss of striatal DARPP-32 and D1R, but not D2R expression (Fig. 6D,E) and the decline in Rotarod performance elicited by cortical mtHtt expression in Glu-$CB1R^{-/-}$ mice (Fig. 6F). As an additional functional readout, cortical mtHtt expression in Glu-$CB1R^{-/-}$ mice was found to abrogate the characteristic stimulant-like pattern on motor reactivity evoked by acute administration of the D1R-selective agonist SKF-81297 (1 mg/kg, i.p.), which points to an impairment of direct-pathway striatal circuitry (Fig. 6G). Of note, MK-801 treatment was able to rescue this effect (Fig. 6G).

Taken together, these data support that CB1R located on corticostriatal projections protects D1R-MSNs from cortical mtHtt-evoked damage by inhibiting glutamatergic transmission.

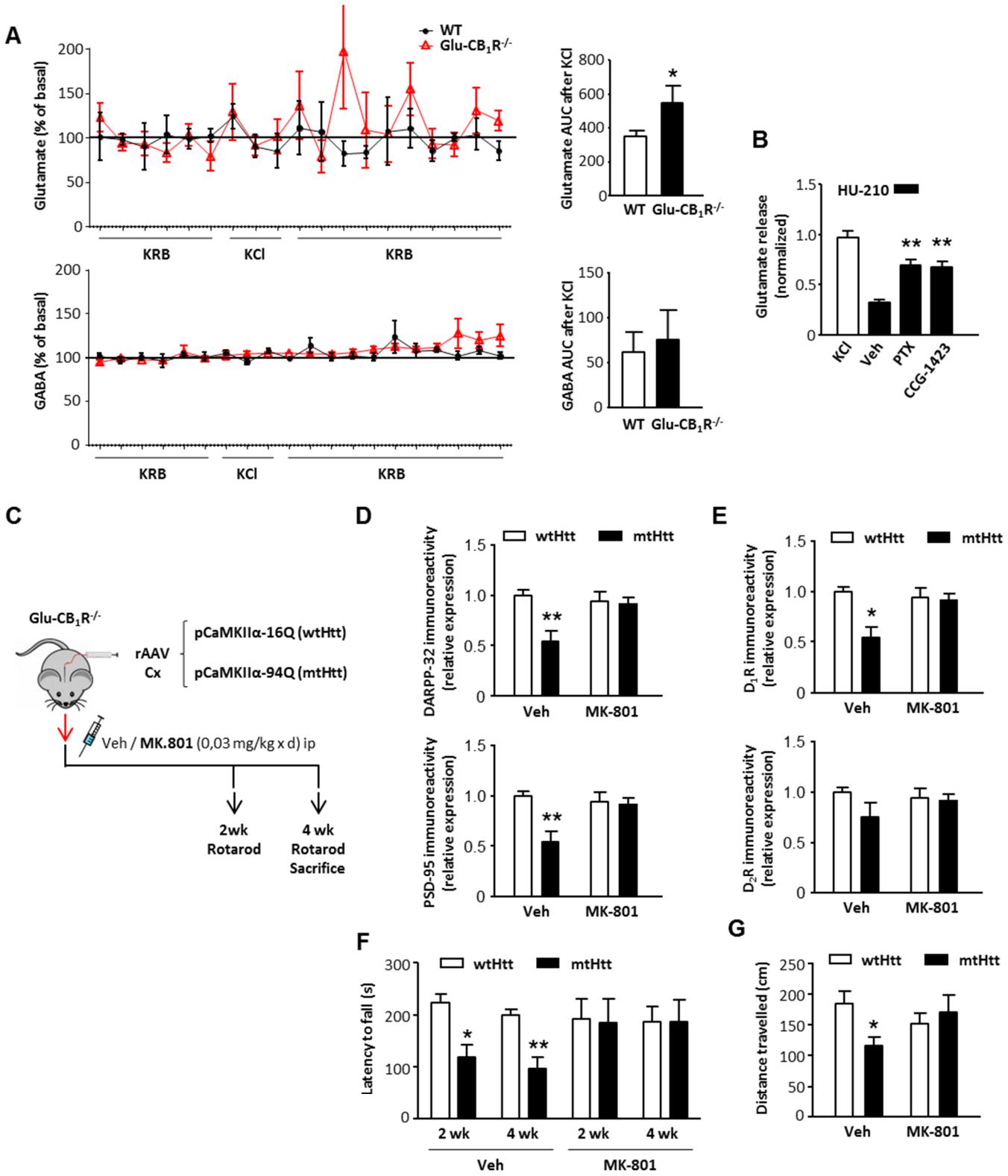

Figure 6. CB1R located on corticostriatal projections protects D1R-MSNs from cortical mutant huntingtin-elicited damage by inhibiting glutamatergic transmission A, Glu-CB1R$^{-/-}$ mice and CB1R$^{floxed/floxed}$ littermates were subjected to microdialysis experiments. Glutamate and GABA concentration was measured in the dorsal-striatum dialysates. Data are presented as percentage of basal glutamate release of each experiment . *p<0.05, **p<0.01 from the corresponding CB1R$^{floxed/floxed}$ group by two-way ANOVA (n = 6). B, Glutamate release from striatal synaptosomes after incubation with no additions, pertussis toxin (1,5 mg/ml, 2 h) or CCG-1423 (25 μM, 45 min) prior to the addition of HU-210 (5 μM). Diagrams show glutamate release induced by KCl (10mM) normalized to release upon KCl stimulation after incubation with each drug. *p<0.05, **p<0.01 from the corresponding vehicle group by two-way ANOVA (n = 6-13). C-G, Glu-CB1R$^{-/-}$ mice and CB1R$^{floxed/floxed}$ littermates (not shown in the figure) were injected stereotactically into the motor cortex with rAAV vectors encoding wtHtt (16Q-CFP) or mtHtt (94Q-CFP) under the control of a CaMKIIα promoter. Animals were subsequently treated with vehicle or MK-801 (0.03 mg/kg/d, i.p.) for 4 weeks. C, Scheme of the experiment. D, DARPP-32 and PSD-95 immunoreactivity in the dorsal striatum. E, D1R and D2R immunoreactivity in the dorsal striatum. In D-E, data are expressed as relative values of the wtHtt-vehicle group. F, Rotarod performance (time to fall), data are presented as mean ± SEM. G, One day after termination of chronic pharmacological treatments and Rotarod assays, ambulation (total distance traveled) was determined 30 min after a single injection of SKF-81297 (1 mg/kg, i.p.). Data are presented as distance traveled (centimeters) after SFK-81297 injection, relative to distance traveled after vehicle injection (ambulation). *p<0.05, **p<0.01 from the corresponding wtHtt-vehicle group by two-way ANOVA (n = 5-6).

CB1R located on corticostriatal projections protects MSNs from astroglia-elicited damage by inhibiting glutamatergic transmission

Astrocytes are pivotal elements in the control of brain glutamatergic signaling (Murphy-Royal et al. 2017). In HD, mtHtt aggregates accumulate in astrocytes from patients (Shin et al. 2005) and animal models (Chou et al. 2008; Bradford et al. 2009) of the disease, and preclinical evidence supports that this can contribute to drive disease progression, at least in part through alterations in glutamate homeostasis (Benraiss et al. 2016; Jiang et al. 2016; Meunier et al. 2016; Jansen et al. 2017). In addition, astrocytes express CB1R, metabolize endocannabinoids, and modulate endocannabinergic transmission (Stella 2010; Navarrete & Araque 2010; Han et al. 2012). Hence, we studied the possible role of astroglial CB1R on mtHtt-evoked striatal damage.

For this purpose we first injected into the dorsal striatum of C57BL/6N mice rAAV vectors encoding wtHtt or mtHtt under the control of a minimal glial fibrillary acidic protein (GFAP) promoter, in order to confine its expression to astrocytes (Fig. 7A,B).

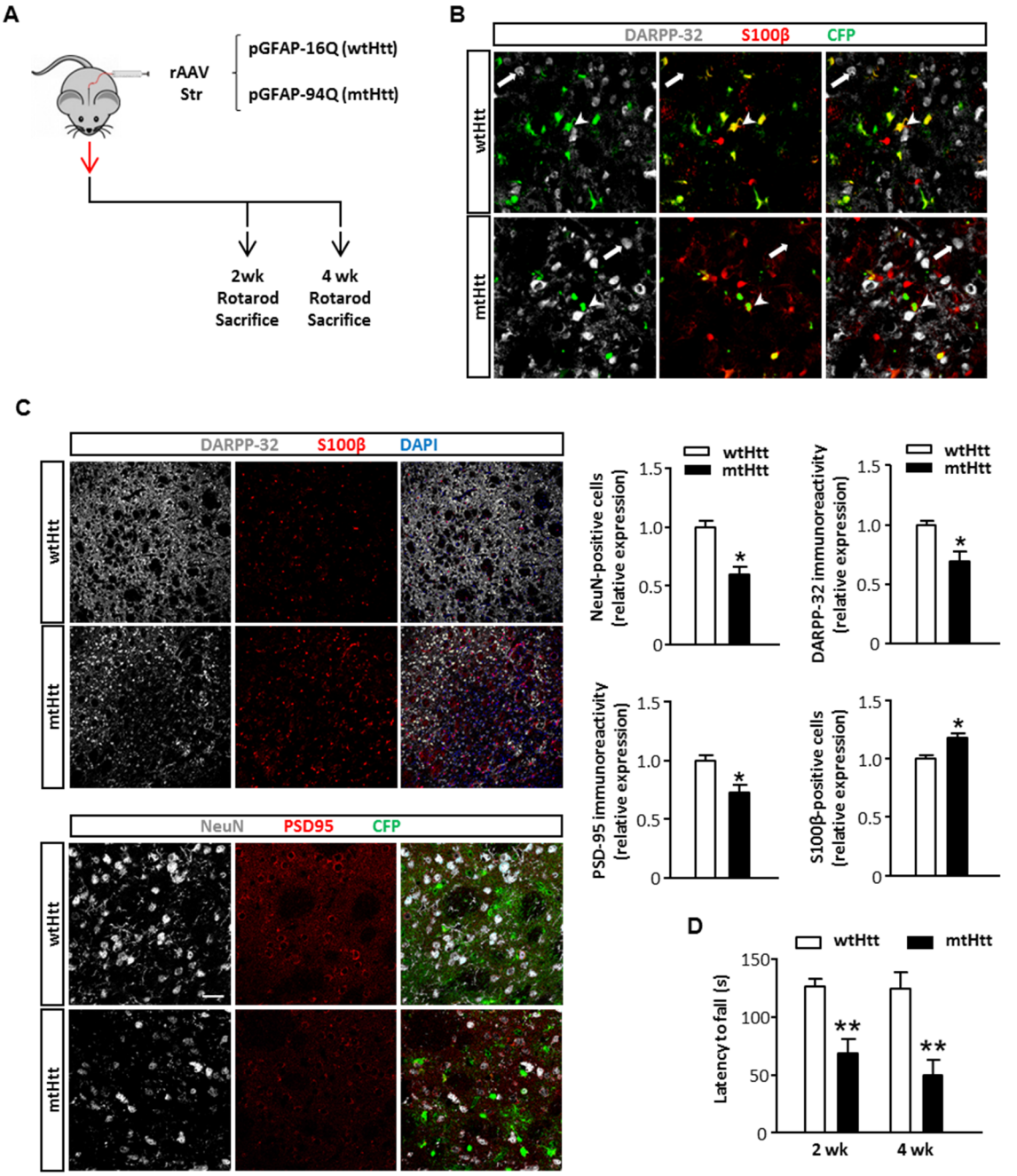

Figure 7. Expression of mutant huntingtin specifically in striatal astrocytes damages MSNs integrity. Eight week-old C57BL/6N mice were injected stereotactically into the dorsal striatum with rAAV vectors encoding wtHtt (16Q-CFP) or mtHtt (94Q-CFP) under the control of a GFAP promoter. A, Scheme of the experiment. B, Expression of the vectors. C, NeuN, DARPP-32, PSD-95 and S100β immunoreactivity in the dorsal striatum. In B-C, data are expressed as relative values of the wtHtt group. Representative images are shown. Scale bar, 50 µm. D, Rotarod performance (time to fall), data are presented as mean ± SEM. *p<0.05, **p<0.01 from the corresponding wtHtt group by unpaired t-Test or Mann Whitney test (n = 4-6) (C) or by two-way ANOVA (n = 10) (D).

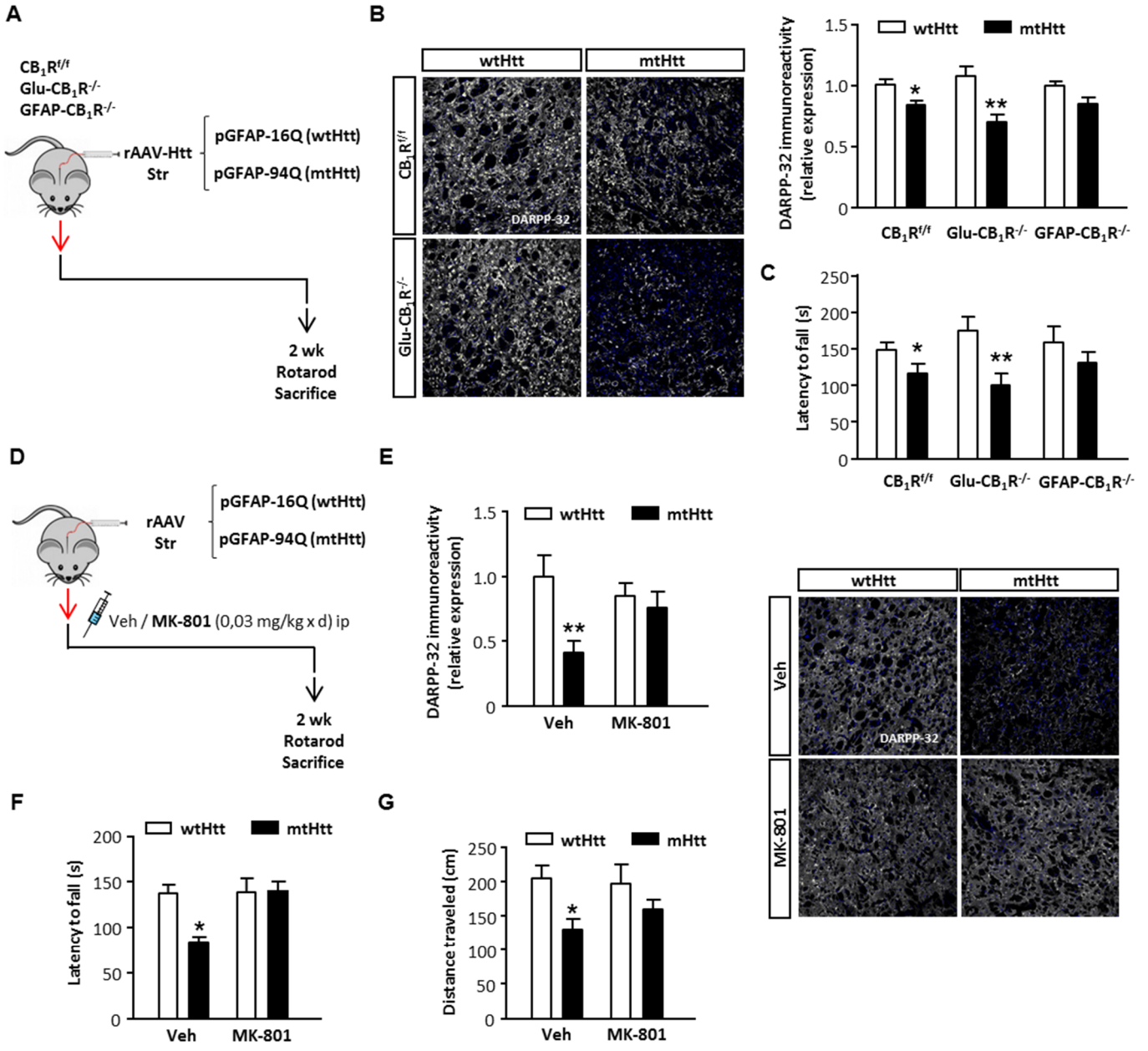

Figure 8. CB1R located on corticostriatal projections protects D1R-MSNs from astroglial mutant huntingtin-elicited damage by inhibiting glutamatergic transmission A-C, GFAP-CB1R$^{-/-}$ mice, Glu-CB$_1$R$^{-/-}$ mice and CB1R$^{floxed/floxed}$ littermates were injected stereotactically into the dorsal striatum with rAAV vectors encoding wtHtt (16Q-CFP) or mtHtt (94Q-CFP) under the control of a GFAP promoter. A, Scheme of the experiment. B, DARPP-32 immunoreactivity in the dorsal striatum. Data are expressed as relative values of the wtHtt-CB1R$^{floxed/floxed}$ group. Representative images are shown. Scale bar, 50 µm. C, Rotarod performance (time to fall), data are presented as mean ± SEM. *p<0.05, **p<0.01 from the corresponding wtHtt group by two-way ANOVA (n = 5-10). D-F, Eight week-old C57BL/6N mice were injected stereotactically into the dorsal striatum with rAAV vectors encoding wtHtt (16Q-CFP) or mtHtt (94Q-CFP) under the control of a GFAP promoter. Animals were subsequently treated with vehicle or MK-801 (0.03 mg/kg/d, i.p.) for 2 weeks. D, Scheme of the experiment. E, DARPP-32 immunoreactivity in the dorsal striatum, data are expressed as relative values of the wtHtt-vehicle group Representative images are shown. Scale bar, 50 µm. F, Rotarod performance (time to fall), data are presented as mean ± SEM. G, One day after termination of chronic pharmacological treatments and Rotarod assays, ambulation (total distance traveled after) was determined 30 min after a single injection of SKF-81297 (1 mg/kg, i.p.). Data are presented as distance traveled (centimeters) after SFK-81297 injection, relative to distance traveled after vehicle injection (ambulation). *p<0.05, **p<0.01 from the corresponding wtHtt-vehicle group by two-way ANOVA (n = 5-8).

Under these experimental conditions, mtHtt produced, 2 weeks after viral injection, a detectable loss of NeuN, DARPP-32, and PSD-95 expression, in concert with astrogliosis (as determined by the astroglial marker S100β) and motor coordination deficits. (Fig. 7C).

To test a possible neuroprotective role of astroglial CB1R in this setting, conditional mutant mice bearing an inducible genetic deletion of CB1R in astroglial cells ($CB1R^{floxed/floxed;GFAP\text{-}CreERT2/+}$ mice; herein referred to as GFAP-$CB1R^{-/-}$ mice), together with their $CB1^{floxed/floxed}$ control littermates, were injected with GFAP promoter-driven wtHtt or mtHtt-expressing vectors into the dorsal striatum (Fig. 8A). Selective CB1R genetic inactivation in astroglial cells did not sensitize MSNs to damage induced by astrogial mtHtt expression (Fig. 8B,C). In contrast, when mtHtt expression was achieved in striatal astrocytes of Glu-$CB1R^{-/-}$ mice, we found that selective CB1R genetic inactivation in cortical principal neurons exacerbated the decline of striatal DARPP-32 expression and motor coordination performance (Fig. 8B,C).

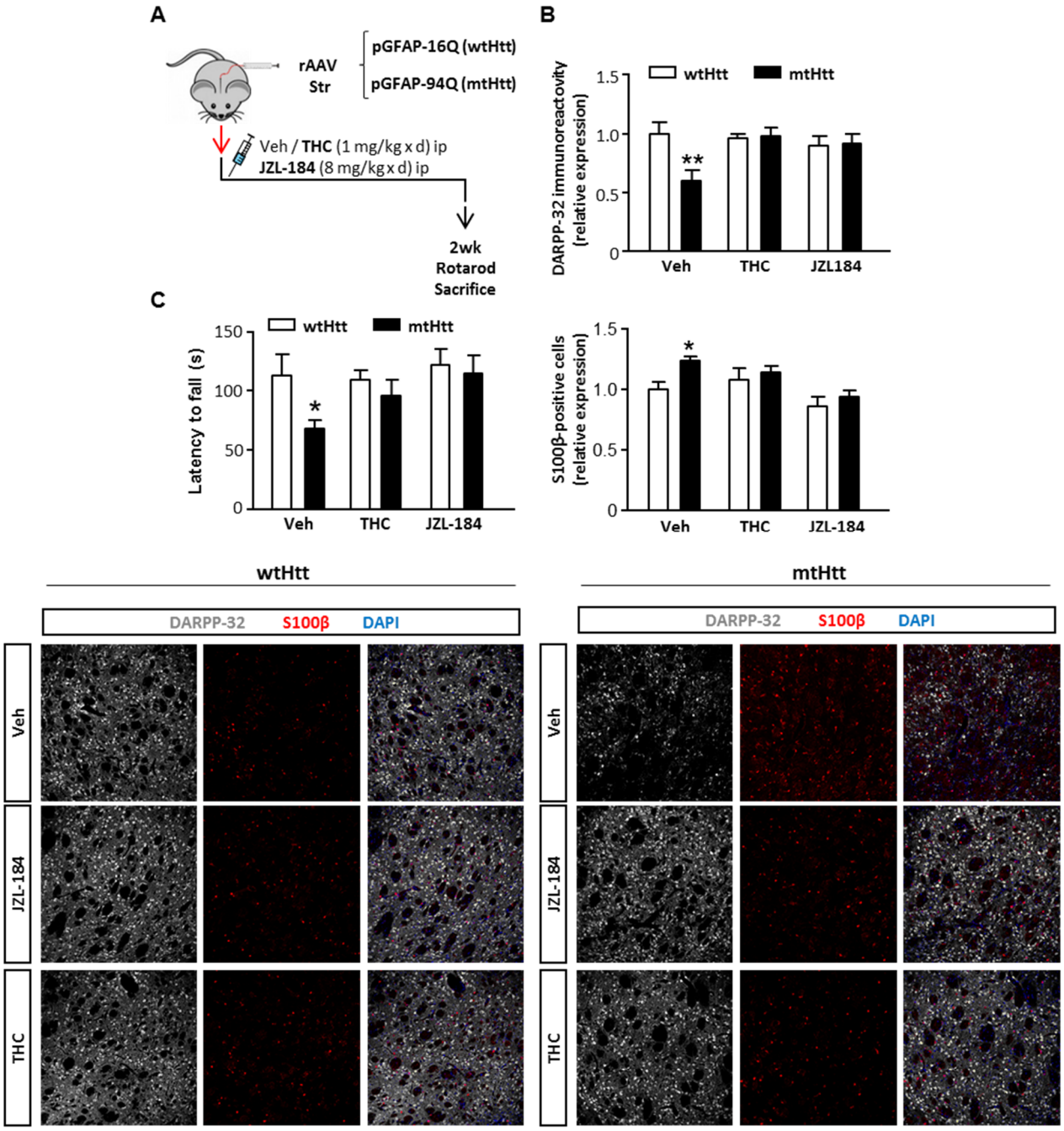

Figure 9. Pharmacological blockade of MGL protects MSNs from astroglial mutant huntingtin-elicited damage Eight week-old C57BL/6N mice were injected stereotactically into the dorsal striatum with rAAV vectors encoding wtHtt (16Q-CFP) or mtHtt (94Q-CFP) under the control of a GFAP promoter. Animals were subsequently treated with vehicle, JZL-184 (8 mg/kg/d, i.p.) or THC (1 mg/kg/d, i.p.) for 2 weeks. A, Scheme of the experiment. B, DARPP-32, and S100β immunoreactivity in the dorsal striatum. Data are expressed as relative values of the wtHtt-vehicle group. Representative images are shown. Scale bar, 50 µm. C, Rotarod performance (time to fall), data are presented as mean ± SEM. *p<0.05, **p<0.01 from the corresponding wtHtt-vehicle group by unpaired t-test (n = 5-8).

We next tested whether cortical CB1R protects MSNs from astroglial mtHtt-induced damage by blunting glutamatergic signaling. For this purpose we injected C57BL/6N mice with GFAP promoter-driven wtHtt or mtHtt-expressing vectors into the dorsal striatum, and treated them for 2 weeks with vehicle or MK-801 (0.03 mg/kg/d, i.p.) (Fig. 8D). MK-801 administration effectively rescued the loss of striatal DARPP-32 (Fig. 8E), the decline in Rotarod performance (Fig. 8F) and the impairment of SKF-81297-induced hyperactivity elicited by mtHtt expression in striatal astrocytes (Fig. 8G).

Taken together, these data support that CB1R located on corticostriatal projections, by inhibiting glutamatergic transmission, protects MSNs, not only from cortical mtHtt-evoked damage, as shown above, but also from astroglial mtHtt-evoked damage.

MGL located on striatal astrocytes fine-tunes neuroprotective endocannabinoid signaling

The experiments described above support that astroglial CB1R does not protect MSNs from mtHtt-induced damage. However, other endocannabinoid system elements located on astrocytes could modulate neuroprotective signaling in corticostriatal circuits. Specifically, as astrocytes are a key site for endocannabinoid degradation (Walter et al. 2004; Uchigashima et al. 2011; Viader et al. 2015) and 2-AG is the main endocannabinoid involved in corticostriatal circuit neuromodulation (Uchigashima et al. 2007; Tanimura et al. 2010), we asked whether astroglial monoacylglycerol lipase (MGL; the main enzyme that deactivates 2-AG) could fine-tune a neuroprotective endocannabinoid tone. For this purpose, we first injected GFAP promoter-driven wtHtt or mtHtt-expressing rAAV vectors into the dorsal striatum of C57BL/6N mice, and treated them for 2 weeks with vehicle or the MGL-selective inhibitor JZL-184 (8 mg/kg/d, i.p.). The cannabinoid receptor agonist -Δ^9-tetrahydrocannabinol (THC), the main bioactive ingredient of cannabis, was used in parallel (at 1 mg/kg/d, i.p.). MGL pharmacological blockade, as well as cannabinoid receptor pharmacological stimulation, prevented the deleterious effects elicited by astroglial mtHtt expression on striatal markers and motor coordination-related performance (Fig. 9B,C).

To evaluate more directly the role of astroglial MGL, conditional mutant mice bearing a genetic deletion of MGL in astroglial cells ($MGL^{floxed/floxed;GFAP\text{-}Cre/+}$ mice; herein referred to as GFAP-$MGL^{-/-}$ mice), together with their $MGL^{floxed/floxed}$ control littermates, were inoculated GFAP promoter-driven wtHtt or mtHtt-expressing vectors into the dorsal striatum (Fig.10A). Selective genetic inactivation of astroglial MGL prevented the deleterious effects evoked by mtHtt expression in striatal astrocytes (Fig. 10B,C).

Taken together, these data support that MGL located on striatal astrocytes controls the availability of a 2-AG pool that ensues protection of MSNs, conceivably by engaging CB1R located on corticostriatal terminals.

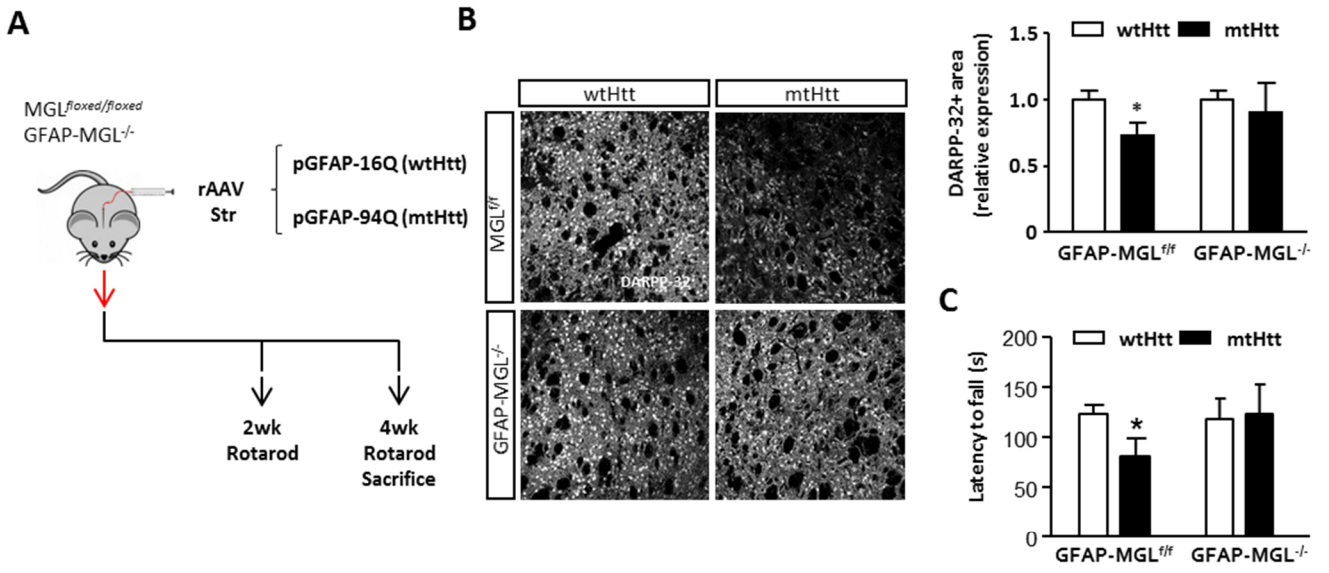

Figure 10 Genetic inactivation of astroglial MGL protects MSNs from astroglial mutant huntingtin-elicited damage GFAP-$MGL^{-/-}$ mice and GFAP-$MGL^{floxed/floxed}$ littermates were injected stereotactically into the dorsal striatum with rAAV vectors encoding wtHtt (16Q-CFP) or mtHtt (94Q-CFP) under the control of a GFAP promoter. A, Scheme of the experiment. B, DARPP-32 immunoreactivity in the dorsal striatum. Data are expressed as relative values of the wtHtt-$MGL^{floxed/floxed}$ group. Representative images are shown. Scale bar, 50 µm. C, Rotarod performance (time to fall). *p<0.05, **p<0.01 from the corresponding wtHtt-$MGL^{floxed/floxed}$ group by two-way ANOVA (n = 4-6).

DISCUSSION

Here, we first manipulated MSNs selectively by means of the DREADD technology to unveil how Gq-protein-evoked signaling affects neuronal functionality (Fig. 1). A DREADDi approach was used previously to study the effects of the selective inhibition of D1R-MSNs or D2R-MSNs in the rat dorsomedial striatum by expressing hM4Di in a herpes virus vector with promoter elements for dynorphin or enkephalin, respectively (Ferguson et al. 2011) CNO administration did not change acute locomotor responses to amphetamine, but altered behavioral plasticity associated with repeated drug treatment (Ferguson et al. 2011). A similar approach found that the hM4Di-evoked inhibition of D2R-MSNs in the mouse nucleus accumbens enhanced the motivation to obtain cocaine (Bock et al. 2013).

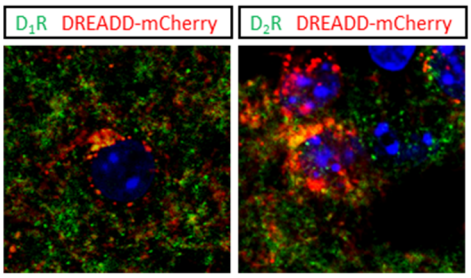

Figure 1. DREADD-mCherry expression in D1R- and D2R-MSNs

Collectively, these and other related studies demonstrate that chemogenetic manipulation of MSNs with DREADDs (Farrell et al. 2013; Ferguson et al. 2013), DREADDi (Ferguson et al. 2011; Bock et al. 2013; Ferguson et al. 2013) or DREADDq (the present study) is a viable tool to assess the impact of specific G-protein-mediated signals on the conceptually proposed opposing roles of the direct and indirect striatal pathways. Moreover, the lack of effect of MSN inhibition (via DREADDi) on acute locomotor responses (Ferguson et al. 2011) compared with the remarkable effects of MSN activation via DREADDs (Ferguson et al. 2013) or DREADDq (the present study) points to a hierarchical subordination of inhibitory to stimulatory metabotropic pathways in simple behavioral tasks.

Likewise, Gq, Gs, and Gi-coupled DREADD-mediated manipulation of the circadian pacemaker in the mouse suprachiasmatic nucleus showed a prominent role of the Gq axis over Gi (and Gs) signaling in controlling circadian rhythms (Brancaccio et al. 2013). In contrast, significant—and opposing— effects of Gq and Gi signaling per se were found upon the chemogenetic manipulation of, for example, mouse agouti-related protein-expressing neurons (Krashes et al. 2011) and calcitonin-gene-related peptide-expressing neurons (Carter et al. 2013) in the control of feeding behavior. Therefore, it is conceivable that the actual relative strength of Gq, Gs, and Gi signals to control neural activity varies significantly among different brain regions and biological processes in vivo.

The precise metabotropic mechanisms involved in the control of the integrity and function of MSNs are not fully understood. A large body of evidence supports that cAMP-dependent cascades are highly relevant and, in fact, both DREADDs and DREADDi alter neuronal activity and plasticity in striatal circuits through changes in cAMP production (Ferguson et al. 2011; Bock et al. 2013; Farrell et al. 2013; Ferguson et al. 2013). Activation of Golf-coupled D1R in direct-pathway MSNs engages multiple signaling pathways, such as PKA, ERK, and CREB, by increasing cAMP production, whereas activation of Gi-coupled D2R in indirect-pathway MSNs leads to downregulation of these cascades (Girault 2012; Cahill et al. 2014). Other Gs-coupled receptors (e.g., A2AR, which is mostly located in striatopallidal MSNs) and Gi-coupled receptors (e.g., CB1R, which is highly enriched in the terminals of both striatonigral and striatopallidal MSNs) make a major contribution as well to tuning the functioning of basal ganglia circuits via cAMP and other related intracellular signals (Kreitzer 2009; Girault 2012).

The class I metabotropic glutamate receptors mGlu1 and mGlu5 are the most relevant group of striatal Gq-coupled receptors. Although activation of the ERK pathway in the striatum can be readily achieved by D1R and ionotropic (NMDA) glutamate receptors, mGlu1/5 receptors also activate ERK through Ca^{2+} release from intracellular stores in synergy with D1R, thereby participating, for example, in drug-induced behavioral plasticity (Girault 2012). However, here, by manipulating Gq-evoked activity selectively in MSNs, we unveil that JNK, rather than ERK or other signaling pathways such as PKC and PI3K/Akt/mTORC1, is the key functional effector of the Gq/PLC/Ca^{2+} axis (Fig.2 A, B). Glutamate has been shown to stimulate JNK in striatal neurons (Schwarzschild et al. 1997) and to cooperate with dopaminergic signaling (mostly via D1R) to induce MSN excitotoxicity (McLaughlin et al. 1998; Tang et al. 2007; Paoletti et al. 2008; J. Y. Chen et al. 2013). Moreover, cocaine (Go et al. 2010) and methamphetamine (Jayanthi et al. 2002) administration, by overstimulating D1R-mediated dopaminergic signaling, induces MSN death and striatal damage at least in part via JNK, an effect that is favored by activation of class I mGlu receptors (Jayanthi et al. 2002; Go et al. 2010).

Therefore, our data align well with prior evidence and strongly support the notion that the JNK cascade plays a pivotal converging role in mediating the malfunctioning of striatal circuits that occurs after overactivation of glutamatergic and dopaminergic

transmission(J. Y. Chen et al. 2013; Cahill et al. 2014). Specifically regarding HD, the involvement of JNK in mutant huntingtin-mediated striatal neurotoxicity is supported by a number of *in vitro* and *in vivo* studies

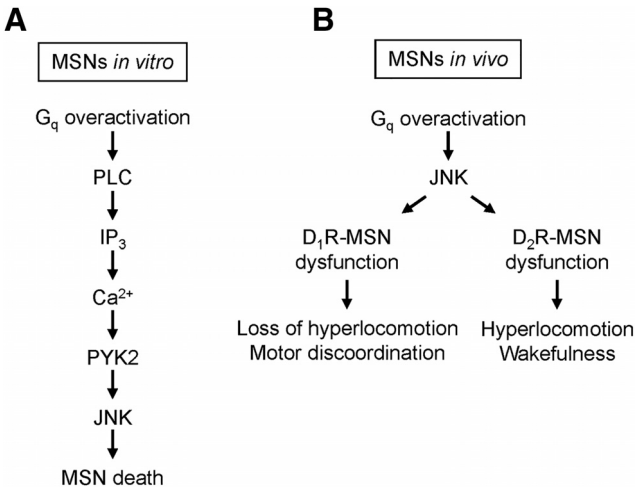

Figure 2. Proposed mechanism of action and impact of sustained Gq-protein signaling on striatal circuitry. A, Gq-protein-coupled signaling upon hM3Dq overactivation in MSNs in vitro induces cell death via a PLC/Ca^{2+}/PYK2/JNK pathway. B, Gq-protein-coupled signaling upon hM3Dq overactivation in MSNs in vivo induces neuronal dysfuction via JNK, which translates into loss of hyperlocomotion and motor discoordination (upon D1R-MSN dysfunction) or into hyperlocomotion and wakefulness (upon D2R-MSN dysfunction).

(Apostol et al. 2008; Perrin et al. 2009; Taylor et al. 2013). Although we are aware that the primary mechanisms underlying the sustained Gq-protein-induced toxicity of MSNs and the DTA-induced toxicity of MSNs are conceivably distinct, pilot experiments conducted in our STHdh cell cultures show that DTA-induced death also seems to be JNK dependent, as shown by the preventive effect of SP600125 (unpublished observations).

PYK2 was originally characterized as a Ca^{2+}-dependent protein kinase that can link elevations of cytosolic free Ca^{2+} concentration (Yu et al. 1996) with JNK activation upon different triggers (Dikic et al. 1996; Tokiwa et al. 1996). PYK2 is highly responsive to neuronal activity. Upon depolarization, it autophosphorylates on tyrosine residues, clusters on postsynaptic densities, and exposes an SH2-binding domain that recruits Src family kinases, thereby activating various signaling pathways (Girault et al. 1999; Bartos et al. 2010). Therefore, PYK2 may connect neuronal activity/plasticity with processes such as neuronal survival and neurite outgrowth/retraction (Girault et al. 1999; Ivankovic-Dikic et al. 2000; Kinoshita et al. 2014) In addition, PYK2 is activated in the rat hippocampus after brain ischemia and kainate-induced convulsions (Tian et al. 2000), which suggests a role for the kinase in the effects of these insults. Therefore, our present findings extend this evidence and specifically show that PYK2 is a novel mediator of Gq/Ca^{2+}-driven striatal dysfunction (Fig. 2A).

In conclusion, the first block of Results of this work sheds new light onto how metabotropic signals control neuronal integrity and functionality. It also supports that the sustained DREADDq-evoked modulation of the direct versus indirect pathway may be adopted as a new tool to understand physiopathological alterations occurring in basal-ganglia-related diseases such as HD, PD, and L-DOPA-induced dyskinesia. For example, regarding HD, the impairment of indirect-pathway circuitry evoked by sustained Gq/JNK signaling seems to recapitulate the dyskinesia/hyperkinesia, as well as the insomnia/reduced REM sleep occurring from early stages of the disease, whereas the impairment of direct-pathway circuitry evoked by sustained Gq/JNK signaling seems to recapitulate the bradykinesia/parkinsonism occurring at later stages of the disease [Fig. 2B (Walker 2007; Arnulf et al. 2008)]. Whether Gq/JNK signaling affects, not only motor behavior, but also other prominent striatal functions such as cognition and motivation may be the subject of future studies.

The second part of this work focused on a key unanswered question in most neurodegenerative diseases: what precise factors dictate the selective vulnerability of a particular neuronal population? In the precise case of HD, the pattern of neurodegeneration is very typical of regional locations as well as neuronal types in the striatum. Thus, MSNs, especially those found in the dorsal striatum (caudate-putamen), represent the main and earliest cell population altered, whereas, for example, striatal interneurons are typically unaffected or only mildly affected at late stages of the disease (Walker 2007). Many studies based on techniques such as PET, autoradiography, and immunomicroscopy have reported reductions in striatal D1R and D2R density from early disease stages in HD patients and animal models. Nonetheless, it is generally believed that D2R-MSNs are affected at earlier stages of the disease and to a greater extent than D1R-MSNs, which is consistent with the notion that early-onset chorea-like movements result from a preferential dysfunction/loss of D2R-MSNs, while later-onset bradykinesia and dystonia are a consequence of an additional dysfunction/loss of D1R-MSNs (Walker 2007; Han et al. 2010; Ross et al. 2014).

Here, we unveil a new key player in this intricate neurochemical scenario by showing that CB1R located on corticostriatal projections, through the control of glutamatergic transmission, dictates a selective protection of D1R-MSNs. CB1R is one of the most abundant metabotropic receptors in the basal ganglia,

where endocannabinoid signaling serves as a major feedback mechanism aimed at preventing excessive presynaptic activity (Glass et al. 2000; Katona & Freund 2008; Atwood et al. 2014). In particular, CB1R is highly expressed on terminals of both D1R-MSNs and D2R-MSNs, where it mediates endocannabinoid-dependent inhibition of GABA release and thus reduction of motor activity (Katona & Freund 2008; Castillo et al. 2012). CB1R is also expressed on glutamatergic terminals projecting from the cortex onto the striatum, thereby blunting glutamatergic output and mediating the so-called endocannabinoid-dependent long-term depression (Gerdeman et al. 2002; Kreitzer 2009). This process was shown to require D2R activation and so was proposed to occur exclusively in D2R-MSNs (Kreitzer & Malenka 2007). However, other findings support that, rather than being specific for D2R-MSNs, endocannabinoid-dependent long-term depression may exhibit a certain preference to occur at D2R-MSNs over D1R-MSNs, and is most likely evoked by different mechanisms in each MSN population (Wang et al. 2006; Bagetta et al. 2011; Mathur & Lovinger 2012; Wu et al. 2015).

On anatomical grounds, different cortical excitatory efferents onto D1R-MSNs and D_2R-MSNs have been proposed: D1R-MSNs seem to receive input preferentially from small, bilateral intratelencephalic projections, while D2R-MSNs seem to receive input preferentially from larger, ipsilateral collaterals of the pyramidal tract (Lei et al. 2004; Raymond et al. 2011). Likewise, excitatory synapses exhibit higher release probability and larger NMDAR currents on D2R-MSNs than on D1R-MSNs (Kreitzer & Malenka 2007).

Overall, these findings suggest that CB1R located on corticostriatal terminals projecting onto D2R-MSNs might blunt physiological glutamatergic transmission preferentially aimed at controlling D2R-evoked dopaminergic control of motor behavior. Concertedly, our results support that CB1R located on corticostriatal terminals projecting onto D1R-MSNs might blunt pathological glutamatergic transmission preferentially aimed at controlling D1R-evoked dopaminergic neurotoxicity (Fig. 3). As D1R is in considerable excess over D2R in the striatum, it is plausible that the former will be more significantly engaged than the latter upon dopamine spillover (Raymond et al. 2011).

On mechanistic grounds, one could speculate that, upon intense activation of intratelencephalic glutamatergic projections, glutamate spillover out of the synapse would evoke on targetted D1R-MSNs the activation of the perisynaptic machinery of endocannabinoid generation, composed of type 1 metabotropic glutamate receptors (mostly mGluR5), heterotrimeric Gq/11 proteins, PLCCβ, and DAGL-α, thus triggering the production of 2-AG (Uchigashima et al. 2007; Katona & Freund 2008), which would retrogradely engage CB1R located on glutamatergic terminals, inhibiting in turn excess excitatory transmission (Castillo et al. 2012) and buffering the neurototoxic effects of extrasynaptic NMDA receptors on D1R-MSNs (Tang et al. 2007; Paoletti et al. 2008; Milnerwood et al. 2010; Okamoto et al. 2009; J. Y. Chen et al. 2013).

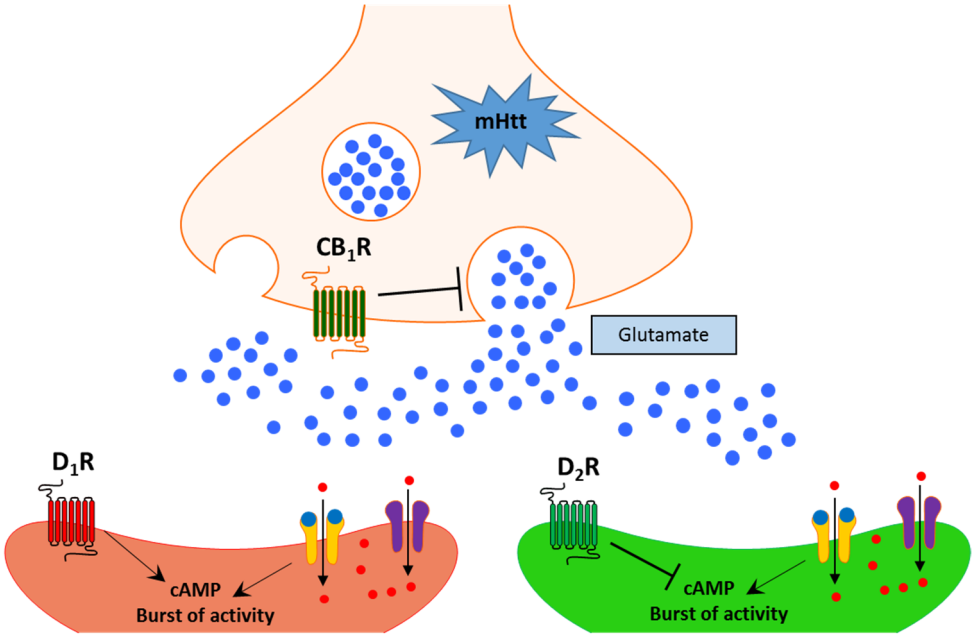

Figure 3. Mechanisms of cannabinoid-induced protection of D1R-MSNs upon mtHtt expression in the cortex. When mHtt-evoked damage occurs specifically in cortocostriatal projection neurons, corticostriatal CB1R (but not astroglial CB1R or MSN CB1R) exerts D1R-MSN protection by blunting glutamate release.

A significant number of studies have dealt with the expression and function of CB1R in HD. As a matter of fact, HD constitutes the best currently available mode disease to assess the pathophysiological relevance and therapeutic potential of CB1R in neurodegenerative diseases. This is due to at least three important reasons: (*i*) CB1R is highly expressed in the striatum at synapses established by neurons containing GABA (especially MSNs, the cells that primarily degenerate in HD) or glutamate (especially corticostriatal projection neurons, which critically control MSN function) as transmitters, and plays a key role in the control of motor behavior (the process that is most characteristically affected in HD) (Katona & Freund 2008; Kreitzer 2009; Castillo et al. 2012). (*ii*) We (Blázquez et al. 2011) and others (Mievis et al. 2011) have demonstrated a neuroprotective role of CB1R in transgenic mouse models of HD; for example, double-mutant mice expressing mtHtt exon 1 in a $CB1R^{-/-}$ background show an overt HD-like phenotype at earlier ages than their single-mutant littermates expressing mtHtt exon 1 in a normal $CB1R^{+/+}$ background. (*iii*) An early and remarkable down-regulation of CB1R expression has been documented in the caudate-putamen of HD patients (Glass et al. 2000) and animal models (Denovan-Wright & Robertson 2000; McCaw et al. 2004), this down-regulation reflecting the damage pattern of MSNs characteristic of the disease; in contrast, the expression and function of CB1R located on corticostriatal projections remains unaffected along HD progression (Chiodi et al. 2012; Chiarlone et al. 2014).

Hence, it is plausible that the maintenance of CB1R on corticostriatal projections constitutes an adaptive mechanism aimed at buffering concerted glutamatergic-dopaminergic excitotoxicity on D1R-MSNs (Fig. 3). Evidence obtained from HD patients and mouse models shows that patterns of communication between cortical projections and MSNs become altered from very early, even asymptomatic stages of the disease, thus indicating that a dysfunctional cortical input to the striatum determines the onset and progression of neurological signs (Thu et al. 2010; Ghiglieri et al. 2012; Unschuld et al. 2012; Estrada-Sánchez & Rebec 2013).

Likewise, it is generally accepted that MSN dysfunction and associated behavioral deficits in HD are caused by the expression of mtHtt not only in MSNs (cell-autonomous toxicity) but also in cortical pyramidal neurons (non-cell-autonomous toxicity) (Gu et al. 2005; Wang, Gray, X. Lu, et al. 2014; Estrada-Sanchez et al. 2015). However, it is still unknown whether these regional features of mtHtt expression define a potential vulnerability of D1R-MSNs *vs.* D_2R-MSNs to damage. Here we show that, in the case of the control of MSN survival by the pool of CB_1R molecules located on corticostriatal projections, cortical pyramidal neurons –but not MSNs or astrocytes- constitute an indispensable site of mtHtt expression for a selective protection of D_1R-MSNs *vs.* D_2R-MSNs to be observed. In addition, when mtHtt is expressed in astrocytes, MGL located in these cells fine-tunes the availability of 2-AG to achieve cortical CB1R-mediated neuroprotection (Fig. 4).

This exciting finding adds to the conceptual view on how non-cell-autonomous mtHtt actions orchestrate complex alterations in the corticostriatal circuit and may help to understand the intrincate pathophysiology of basal ganglia disorders.

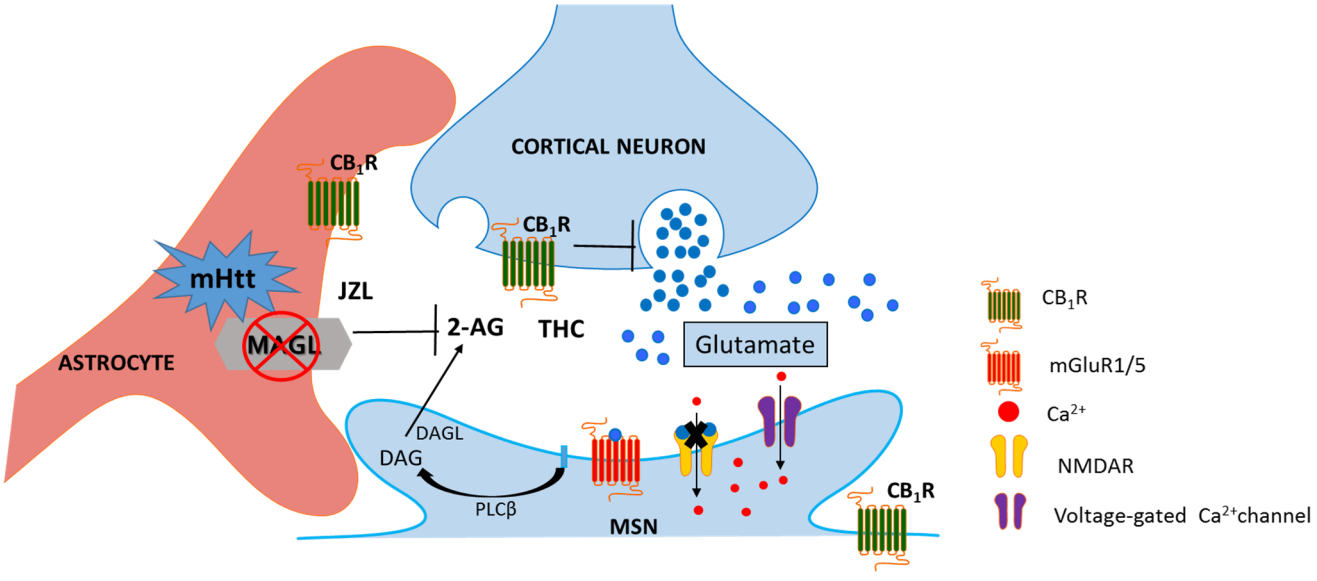

Figure 4. Mechanisms of cannabinoid-induced neuroprotection of D1R-MSNs upon mtHtt expression in astrocytes. When mHtt-evoked damage occurs specifically in striatal astrocytes: (I) Corticostriatal CB1R (but not astroglial CB1R or MSN CB1R) exerts D1R-MSN protection by reducing excitotoxicity. (II) The suppression of astroglial MGL activity also exerts neuroprotection.

CONCLUSIONS

The functionality of MSNs is tightly controlled by various metabotropic receptors. An imbalance in any of these receptors could lead to dysfunction and degeneration of the corticostriatal neuronal network, and may have different consequences depending on the MSN population. CB1R, which is highly expressed in the striatum and plays a key role in its related functions, represents one of these metabotropic signalling systems that determines the differential cellular responses within the corticostriatal system.

The results obtained in this Thesis allow us to obtain the following **CONCLUSIONS**:

I. Striatal circuits can be "turned on" by acute Gq-protein signalling or "turned off" by sustained Gq-protein signalling. Specifically, sustained Gq-protein signalling inactivates MSNs of the dorsal striatum by an intracellular pathway that relies on JNK.

II. CB1R located on corticostriatal projections, mainly by blunting glutamatergic output, selectively safeguards D1R-MSNs of the dorsal striatum.

Collectively, these findings define the molecular mechanism and functional relevance of Gq-protein-driven signals in striatal circuits under normal and overactivated states, and define cortical CB1R as a key neurochemical player in dictating a dissimilar vulnerability of D2R-MSNs vs. D1R-MSNs. Altogether, they may contribute to understand the role of coordinated cannabinergic-glutamatergic signaling in the control of the direct and indirect corticostriatal pathways, and their dysregulation in basal ganglia disorders.

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